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PRINCIPAL INVESTIGATOR: Dr. Priscilla Clarkson

CONTRACTING ORGANIZATION: University of Massachusetts
Amherst, MA 01003

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Dr. Priscilla Clarkson

E-Mail: clarkson@kin.umass.edu

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14. ABSTRACT

Cigarette smoking is prevalent in the military and is associated with increased risk for musculoskeletal injury. This study investigated differences in functional strength and molecular alterations in blood and muscle samples between smokers and non-smokers in response to a muscle-damaging exercise. 10 smokers and 10 non-smokers performed a maximal eccentric exercise of the non-dominant knee extensors. Isometric and isokinetic strength were measured pre- and 5min, 1, 4, and 9d post-exercise. Blood was collected pre- and 20h post-exercise, and biopsy samples were obtained from control and exercise legs at 48h post-exercise. Smokers had greater loss in flexion strength at 4d post-exercise for isometric strength ($p<0.05$), likely due to the effect of the biopsy and slower healing rate in the smokers. Using PCR array, we found that non-smokers increased gene expression (mRNA) while smokers had a downregulation of expression in most of 44 genes studied. Significant changes in mRNA involved several key functions involved in muscle regeneration including maintenance of structure, nitric oxide signaling, angiogenesis, myogenesis, and inflammation, generally showing an attenuated response to exercise in the smokers. For example, regarding inflammation, in smokers there was an attenuated exercise-induced increase in CHUK (IKKα) mRNA, an upstream mediator of nuclear factor-kappa B (NFκB), a key player in muscle inflammation. Further testing showed that the activity of the canonical and non-canonical NFκB pathways was altered in non-smokers but not in smokers. Western blotting yielded significantly higher phosphorylated and total ERK1/2 (involved in several pathways important to muscle regeneration) in smokers at baseline. Taken together, these data suggest that smokers not only have baseline differences in key components of muscle function but also show attenuated responses to eccentric exercise, especially in pathways involved in inflammation and regeneration.

15. SUBJECT TERMS

Smoking; Exercise- induced muscle damage; Strength loss; Molecular Alterations

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THE EFFECT OF SMOKING ON MUSCLE ADAPTATION TO EXERCISE

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INTRODUCTION

BACKGROUND

Survey results from the Centers for Disease Control in 2009 showed that about 46 million people, or 20.6 percent of the adult population in the US, reported smoking cigarettes.[1] Cigarette smoking accounted for about 443,000 deaths (1 in every 5 deaths) per year,[2] making it the leading cause of preventable death.[3-7] Cigarette smoking is associated with a wide variety of chronic diseases and conditions, including increased risk for musculoskeletal injury[8, 9] and prolonged healing.[10, 11]

Wen and colleagues reported higher risk for injury-related death in smokers, with the highest risk following work-related injury.[12] This was a dose dependent risk, with relative risk for injury compared to non-smokers increasing with the number of cigarettes smoked per day so that heavy smokers (>20 cigarettes per day) had a 5.72 times greater chance of death from a work-related injury. Greater risks for injury have been reported in a variety of professions and locales in smokers as compared to non-smokers. In Great Britain, a large survey-based study found that smokers were twice as likely as non-smokers to incur a minor injury or accident at work.[13] Similar risk for injury was reported in smokers in Northern France.[14] Increased risk for injury in smokers has also been found in agricultural workers,[15] construction workers,[16] workers in the manufacturing industry,[17] postal workers,[18] and railway workers.[19] Material-handling workers who smoked were more likely to claim worker's compensation for back injury than former or non-smokers, indicating that smokers may have more prolonged pain and delayed recovery.[20] It is our contention that smokers have an impaired ability to respond to strenuous exercise stress through normal repair mechanisms, thereby making their muscles more susceptible to injury.

Strenuous unaccustomed resistance exercise with muscle lengthening (eccentric) contractions has been used in the laboratory to study transient muscle injury/damage and repair. Following the initial exercise stimulus is a coordinated inflammatory response. One common factor underlying many of the negative health consequences of smoking is an altered internal inflammatory environment.[21-27] However, prior to the results of the study reported herein, it was unknown whether this altered inflammatory environment could impact smokers' response to a muscle exercise stress that induces an inflammatory response. Our results from this study suggest that dysfunctional inflammation and disrupted molecular signaling impair regeneration leading to inadequate muscle repair in smokers, which may explain the increased risk for musculoskeletal injury. Investigating the exercise-induced response to muscle exercise stress provided clues as to

how smoking impacts risk for musculoskeletal injury and prolongs recovery. *The goal of this research was to determine muscle molecular signaling and systemic inflammation at rest and in response to strenuous exercise in smokers and non-smokers.* This study provides a new area of research, offers an innovative approach to solving the serious problem of how smoking contributes to musculoskeletal injury, and can significantly advance both clinical and basic science

MILITARY SIGNIFICANCE

Cigarette smoking is a serious problem in the military.[6,7,19] Not only does smoking affect the cardiovascular health of soldiers, it also, for unexplained reasons, is an independent risk factor contributing to musculoskeletal injuries.[28] Several studies have reported that smokers in the military have a higher risk of musculoskeletal injury than non-smokers.[8-11, 28-30] In fact, cigarette smoking alone was an independent risk factor (compared with other lifestyle factors, including low fitness levels) for training injuries.[28] Altarac et al. [10] examined 2002 Army recruits who were participating in 8 weeks of basic military training and found that, when controlling for all other lifestyle factors, the adjusted odds ratio was 1.5 for injury rates in smokers versus non-smokers.[31] A study of 15,140 US Army personnel hospitalized for common musculoskeletal disorders between 1989-1996, found that smokers were at a greater risk for disability following meniscal injuries.[11] In addition, injured active service members who smoked were more likely to be discharged due to physical disability after musculoskeletal injury.[11]

Smokers also exhibit increased complications and prolonged healing after injury.[9-11, 28] Inadequate muscle repair in smokers could lead to increased vulnerability to injury, particularly following intense exercise stress and during situations requiring a high degree of training.[32] For example, during basic training in the military, new recruits are subjected to intense training. Further, military personnel are often deployed to situations where they are under constant psychological and physical stress as well as high risk for injury.[32] Smoking is a strong part of the military culture, with approximately 50% of active duty military personnel smoking regularly.[33] Smoking could be endangering recruits both during basic training with unaccustomed exercise stress and when deployed. Increased risk for injury has also been reported in current smokers within the military outside of basic training, when they are permitted to smoke.[28]

Our work from this study has found that smoking negatively impacts the muscle's ability to respond to strenuous exercise stress, which may leave the musculoskeletal system vulnerable to injury. These results will be important in finding ways to reduce injury rate in soldiers, especially during basic training when exercise is often novel and unaccustomed.

PUBLIC PURPOSE

A report of NIOSH Scientific Workshop on "Work, Smoking, and Health" held in 2000 (http://www.cdc.gov/niosh/docs/2002-148/pdfs/2002-148.pdf) noted that despite the substantial progress made by the public health and medical communities in combating cigarette smoking, the burden of tobacco-related illness has shifted toward blue-collar and service sector workers, whose cigarette smoking and exposure to many other occupational health and safety hazards is significantly greater than white collar workers. One recommendation from that meeting was to improve understanding of mechanisms of interactions of smoking and other factors, such as

injury. Although the report concluded that smoking increased the risk of injuries and injury-related death, the underlying biological mechanisms are not clear. The results of this study significantly move the field forward and answer important questions regarding how chronic smoking affects skeletal muscle response to stress. Data from our study may explain increased risk for musculoskeletal injury in smokers in the workplace.[13-17, 19] Furthermore, using our unique exercise model, we were able to contribute information that may be useful toward the understanding of mechanisms underlying prolonged healing after surgery and during wound healing, back pain, recovery from injury, and susceptibility to overuse injury in smokers.[8, 10, 34, 35]

BODY

Technical objectives and hypotheses

The objective of this study is to determine the molecular mechanisms underlying the response to resistance exercise in smokers and non-smokers**.** Smokers were defined as smoking ≥ ½ pack of cigarettes per day for 5 years or more. A muscle biopsy was taken 48 hours post-exercise from both the exercised and non-exercised (control) muscle so that we could examine key indicators of 4 important pathways that could contribute to impaired adaptation in smokers: protein synthesis and degradation, regeneration, inflammation, and angiogenesis. We used a knee extension eccentric exercise performed on a Biodex dynamometer to induce muscle stress.

After resistance exercise, we expected that in muscles of smokers compared with non-smokers there would be a blunted increase in:

- *Hypothesis 3.1* levels of phosphorylated AKT with corresponding changes in phosphorylation of the up and downstream targets of AKT as compared to non-smokers
- *Hypothesis 3.2*. gene expression and protein levels of remodeling proteins including collagen IV, desmin, actin, and HSP70
- *Hypothesis 3.3*. inflammatory factors such as TNFα and MCP-1
- *Hypothesis 3.4*. vascular endothelial growth factor (VEGF)

Methods

We recruited 20 healthy men between 18-35 years old, 10 smokers and 10 non-smokers. Smokers were defined as smoking ≥ ½ pack/day for at least 5 years; non-smokers were those who have never smoked regularly and at the time of the study were non-smokers. Subjects were asked to maintain their normal pattern of smoking during the course of the study. Subjects were healthy and had not weight trained or had a job requiring the lifting and lowering of heavy materials for at least 6 months prior to beginning the study. Subjects signed an informed consent document approved by the University and the DOD Institutional Review Boards.

The testing schedule is outlined in Table 1. We used a knee extension eccentric exercise. Strength was assessed at 0°/sec (3 repetitions/1 min between reps), 60°/sec (3 consecutive

repetitions), and 180°/sec (5 consecutive repetitions). We used a previously validated exercise protocol to induce muscle stress.[36] It consisted of 100 maximal isokinetic eccentric contractions at 30°/sec, and was performed on the Biodex dynamometer (Biodex System 4 Pro, Shirley NY). There were 10 sets of 10 repetitions with a 10 sec rest interval between repetitions. Between sets there was a 1 min rest period. An advantage to the use of the Biodex dynamometer is that the protocol, including all rest periods, is pre-programmed and thus will help eliminate variance between subjects. After each eccentric contraction, the leg was moved passively to the starting position.

Table 1. Testing Schedule

Visit 0	Informed consent document administered
Visit 1 (within 2wk of Visit 0)	Subjects reported to the lab fasted (no food, only water) for 8-12 hours. Blood drawn. Familiarization with strength testing and exercise
Visit 2 (≥2d and ≤ 4d of Visit 1)	Strength testing Exercised one leg (non-dominant) Post-exercise strength testing
Visit 3 (20hr after Visit 2)	Subjects reported to the lab fasted for 8-12 hours. Blood drawn. Strength testing
Visit 4 (48hr after Visit 2)	Subjects reported to the lab fasted for 8 hours before the biopsy procedure. Standardized meal and biopsy of both legs
Visit 5 (2d after Visit 4)	Strength testing and biopsy site check
Visit 6 (1 week after Visit 3)	Strength testing, biopsy site check and suture removal

Subjects reported to the lab fasted (no food only water) for 8 hours and were fed a standardized meal (2% milk, juice, and 2 granola bars) 3-4 hours before the biopsy to regulate nutritional influences on the AKT pathway. Biopsies from both the non-exercised (control) and exercised leg were taken at 48 hours post-exercise. We chose to use the non-exercised leg as the control leg to eliminate the potential effects of a second biopsy on the same leg within 48 hours. The percutaneous needle muscle biopsy was obtained from vastus lateralis muscles using a Bergstrom 5-6 mm biopsy needle. Skin was first lightly anesthetized with 4 ml of 2% lidocaine hydrochloride solution, a small (1-3cm) incision was made in the skin and fascia, the biopsy needle was inserted, and about 200 mg of tissue was removed and rapidly frozen in liquid nitrogen. Samples to be used for immunohistochemistry were mounted in embedding media (O.C.T, Miles INC. Elkhart, IN) and rapidly frozen in liquid nitrogen. All samples were stored at –80^{o}C until analysis.

In addition to muscle biopsies, we also collected blood samples from the subjects at visits 1 and 3. For these visits, we asked subjects to report to the lab fasted overnight for 8-12 hours and took approximately 1-2 tablespoons of blood via venipuncture of the antecubital space. The samples from visit 1 served as a baseline (pre-exercise) sample, and the samples collected at visit 3 were taken 20 hours post-exercise. We used these blood samples to investigate potential differences in

the levels of inflammatory cytokines between smokers and non-smokers following eccentric exercise.

Key Research Accomplishments

Institutional Review Board Approvals

We submitted IRB applications to the University human subjects board (IRB) and Army Human Research Protection Office (HRPO). Approval was received by the IRB on 1 December 2008. After revision by request of the HRPO, we received approval from the HRPO on 2 October 2009. We subsequently received approvals for amendments made to original study documents: informed consent, flyers, telephone screen form, and protocol. The flyer was amended four times, the latest version approved on 20 April 2010. The third and fourth amendments were designed to recruit smokers only, as we had completed data collection of non-smokers. For the fourth and final version we consulted a smoker as to its effectiveness in drawing his (a smoker's) interest. The informed consent was modified five times and the protocol modified three times, with the final versions approved on 1 February 2010. Alterations were primarily typographical in nature or wording changes for clarification. Those that affected the methodology of the study including the following: exercise performed at 30 degrees per second rather than 90, the addition of strength testing at visit 3, visit 5 occurred two days post-biopsy rather than one, the addition of blood collection for cytokine measurement at visits 1 and 3, and strength testing/exercise on the non-dominant leg only. All staff members were required to complete training in lab safety and human subjects research as required by the university. Staff members were all certified in human subjects testing.

Organizing Tasks

Other tasks necessary to organizing the study were performed. Data collection books (case report forms, CRFs—**Appendix A**) were designed. CRF binders were created and the investigator's notebook prepared. The CRFs were then modified as necessary during pilot testing (see below). A labeling system and sample collection organization chart were created for blood and biopsy samples. Standard operating procedures (SOPs) for blood and muscle sample collection, and strength testing and exercise on the Biodex were written and implemented (**Appendix B**). All staff members were trained on data collection techniques, including strength testing and exercise on the Biodex, and blood and biopsy collection procedures. Prior to testing subjects each staff member was required to complete data collection for strength measures and exercise on pilot subjects, and the data were analyzed. All staff members were observed by an experienced tester and the data were scrutinized for variability among repetitions.

Pilot Subject Testing

Before we began recruiting subjects, we tested three pilot subjects for all procedures except blood draws and biopsies. Two of those subjects were tested on both the dominant and non-dominant leg. After analyzing the data, we found a large discrepancy between the dominant and non-dominant legs for strength loss. From this, we determined that it was necessary to modify the protocol to only test the non-dominant leg. By testing either leg, variability would have been much higher. Because we are not utilizing a crossover design, limiting the exercise and strength testing to the non-dominant leg reduced variability.

Recruitment

Subject recruitment was done primarily through posting flyers on the University of Massachusetts Amherst campus and surrounding community. Smaller flyers were placed on tables in the University dining commons and Campus Center restaurant area. To recruit smokers, flyers were focused in locations likely to have a higher population of smokers, such as public ashtrays and break areas. Small flyers were handed individually to smokers seen in public areas. Through March 2010 flyers were posted in the towns of immediately surrounding the University. Flyers were also posted in establishments with license to sell cigarettes within the town of Amherst. Beginning April 2010 flyers were also posted in towns further removed from the University. Towns with a higher population of smokers, such as those near Springfield, MA, were targeted. A total of over 100 hours was spent flyering the community. Flyers were included in an issue of a local newspaper that reaches over 11,000 patrons. Emails with the study flyer were sent to the University graduate students and the University Civil Engineering department.

When a potential subject contacted us, whether by email or telephone, we returned the contact as quickly as possible, usually within an hour of receipt. We replied to all emails with general information and attempted to contact those individuals by telephone. If we did not hear back from a potential subject after the initial information was sent, we contacted him again, twice. During telephone calls we provided study information and, if the individual was interested, performed a telephone screen. If the subject passed the screen, we scheduled him for an orientation visit. We contacted all potential subjects by telephone the day before the scheduled orientation visit to verify the study appointment and interest in participating.

The study coordinator performed the orientation visits, often with a staff member assisting. At the orientation the informed consent was discussed in detail, the biopsy procedure was explained, and the potential subject was shown the biopsy needle. The individual was questioned further to verify that he matched the study criteria. If the subject passed the orientation visit and was still interested in participating, he was scheduled for the remainder of the study visits. He was contacted by telephone two days before visit 1 to remind of compliance requirements (no alcohol, caffeine, anti-inflammatory drugs) and to verify the study schedule. If the potential subject did not report to the laboratory at the scheduled time, he was contacted again with an attempt to reschedule. This occurred four times for non-smokers and 17 times with smokers. Overall, smokers were a greater challenge to recruit and retain than non-smokers.

On 24-May-2010 the final subject passed telephone screening and on 22-June-2010 he was enrolled in the study. Overall, 43 non-smokers and 82 smokers were screened by telephone to enroll a total of 10 non-smokers and 13 smokers; three smokers were disqualified after enrolling in the study. The reason these subjects were disqualified was failure to report to the laboratory for required study visits. In one case, the subject had not yet performed the exercise and was scheduled to continue with the study; however, he proved to be unreliable in his ability to attend study visits and after numerous attempts to reschedule his visits he was disqualified. In the two other cases, the subjects attended all visits through the third visit (1d post-exercise) but did not show up for the biopsy visit and could not be contacted with multiple attempts. Due to the study design, these subjects could not continue and were therefore disqualified. In Table 2 contacts, screening, and enrollment are presented for smokers and non-smokers. Note that it took 3 months to complete enroll and complete the non-smokers and 7 months to enroll and complete the smokers.

Table 2a: Recruitment log by month including initial contacts and telephone screening.

Month	Calls	Emails	Passed Screen	Failed Screen	Not Interested	Orientation Scheduled
Non-Smokers						
January	33	36	8	16	45	8
February	20	56	5	10	61	5
March	6	6	1	3	8	1
April	1	0	0	0	1	0
May	N/A	N/A	N/A	N/A	N/A	N/A
June	N/A	N/A	N/A	N/A	N/A	N/A
Total	**60**	**98**	**14**	**29**	**115**	**14**
Smokers						
January	7	5	1	9	2	1
February	9	17	1	11	14	1
March	14	11	6	9	10	5
April	21	4	8	6	11	8
May	38	0	18	4	16	16
June	13	5	6	3	9	3
Total	**102**	**42**	**40**	**42**	**62**	**34**

Table 2b: Recruitment log by month from orientation to study end-point.

Month	Orientation Scheduled	Enrolled	No Show at Orientation	Drop Out or Disqualified	Completed Study
Non-Smokers					
January	8	6	2	0	6
February	5	3	2	0	3
March	1	1	0	0	1
April	0	0	0	0	0
May	0	0	0	0	0
June	0	0	0	0	0
Total	**14**	**10**	**4**	**0**	**10**
Smokers					
January	1	0	1	0	0
February	1	0	1	0	0
March	5	2	2	1	1
April	8	1	6	0	1
May	16	9	7	2	7
June	3	1	0	0	0
July	0	0	0	0	1
Total	**34**	**13**	**17**	**3**	**10**

The last non-smoker finished testing on 20-May-2010 and the final smoker completed his visits on 27-July-2010. Adequate biopsy samples were obtained from both control and exercise legs for all subjects, a total of 40 biopsies (20 subjects, one biopsy per leg) and were placed in long-term storage with liquid nitrogen until RNA and protein assays were optimized.

Significant Adverse Event

One subject experienced a significant adverse event, likely caused by the study. The subject was hospitalized with muscle pain and swelling and was diagnosed with muscle hematoma. The subject remained in hospital for 3 days under observation and after release attended physical therapy sessions to regain muscle strength. The evening of hospital admission and each day after the study coordinator contacted the subject by telephone or email. The coordinator also visited the subject while the subject was in hospital. After the subject was discharged from hospital, the coordinator continued to follow up at least once a week. The subject completed physical therapy and returned to a normal level of function. This adverse event was reported to both the University IRB and the HRPO. The medical monitor, Dr. Pierre Rouzier, reviewed the case and determined that the risk was rare and stated in the informed consent. He felt the treatment was appropriate and the outcome was positive. Dr. Rouzier recommended that we continue to follow up with the subject and the study be allowed to continue. No other major adverse events occurred in the study. Gene expression analysis was performed on this subject's muscle biopsy samples. These data are presented below (***Reportable Outcomes***, pages 31-36).

Data Entry

Subject data pertaining to strength testing and exercise was entered by hand into a database. It was necessary to enter these data by hand due to the limitations of the Biodex equipment. Two different individuals entered all of the data, and a data audit was performed to check data between the two databases. While analyzing the data, we discovered that the Biodex software was incorrectly analyzing data from the exercise bout. Therefore, a staff member was assigned to manually measure and re-enter data output from the exercise session only. In addition, we re-assessed the isometric and isokinetic strength data at all timepoints and found that the Biodex had interpreted those data correctly. Approximately 400 hours was spent in total entering data.

Molecular Assays

Once collected from the subject, each biopsy sample was split into 3 separate portions for later analysis by the following methods; RNA of candidate genes will be analyzed by qRT-PCR and proteins by Western Blotting (WB). Analysis by these methods limits the number of genes to be analyzed, which are chosen based on the literature. To be the most time- and cost-efficient, we used a targeted PCR array (SABiosciences, Frederick, MD), where we analyzed 44 genes, plus controls, at one time. The PCR arrays were completed in January, 2011 and data analyzed using a repeated measures ANOVA (see ***Reportable Outcomes***, below). RNA isolation was optimized and samples completed in October, 2010.

Based on the PCR array data, protein was isolated from nuclear and cytosolic fractions. By doing this, we could analyze activity of several proteins that act by translocating to the nucleus. Using the Western blot technique we sought to follow up genes of interest determined from the PCR array analysis to see if there is a significant difference in the protein content of these genes. This

analysis would reveal whether the significant differences in mRNA (from PCR) were also found at the protein level (i.e. that the mRNAs were translated into protein).

We next isolated protein from a second muscle sample without separating into fractions to analyze those proteins that do not translocate to the nucleus. Western blotting was performed to determine ERK1/2 and AKT activity (measured by phosphorylation status) and exercise-induced changes of protein levels for desmin, α-actin, collagen 4, fibroblast growth factor (FGF), caspase-3, F-box only protein 6 (FbxO6), and matrix metalloproteinase-9 (MMP9) (see ***Reportable Outcomes***, below).

Based on findings from the PCR arrays, we chose to investigate nuclear factor kappa B (NFκB) activity in the nuclear fractions using DNA-binding ELISAs (Active Motif, Carlsbad, Ca). By using antibodies against p65, Rel-B, and c-Rel, we sought to determine differences in activity of several NFκB pathways (see ***Reportable Outcomes***, below).

REPORTABLE OUTCOMES

Biodex Strength Measures

Statistics

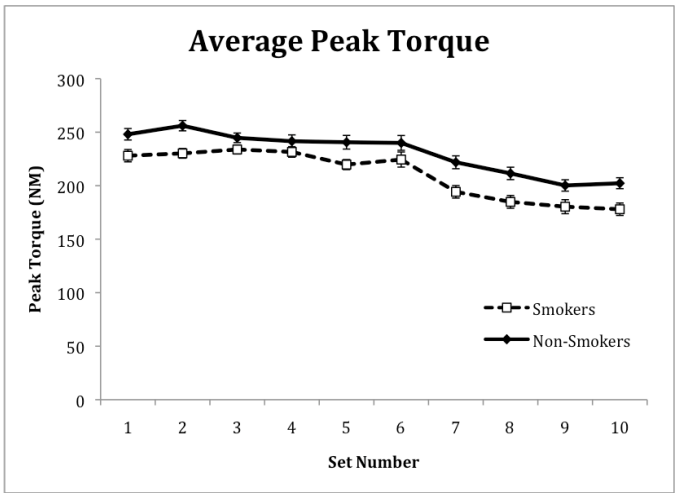
Data were analyzed by repeated measures ANOVA and post-hoc testing with Tukey-Kramer using SAS statistical software for some of the strength measures. Due to the relatively small sample size, we are defining $p<0.05$ as significant and $p<0.10$ as a trend.

There was no significant difference between smokers and non-smokers for baseline isometric knee extension strength. Smokers had lower baseline strength than non-smokers for knee extension at 180°/sec (84.7%, $p<0.05$) and knee flexion at 60°/sec (72.8%, $p<0.01$) and 180°/sec (71.8%, $p<0.01$). Therefore, baseline strength was included as a covariate when performing the statistics on these measures. The data consist of smokers (N=10) and non-smokers (N=9; the subject with the adverse event [see above] was removed from the graph and the analysis). A description of the results appears above each of the following graphs.

Exercise Session

During exercise, total work did not differ between non-smokers and smokers. The following four figures are visualizations of the data by repetition collected from the exercise session consisting of average peak torque, average work done, average power, and time to peak torque. The X-axis refers to the 10 sets of 10 contractions/set at 30°/sec. Beside each graph are the p-values from the repeated measures ANOVA with the main effects for Time (pre and post-exercise measures) and Group (Smokers vs Non-Smokers) and the interaction term of Time by Group (T*G) (ns indicates not significant).

Below is the average knee extension peak torque during exercise. Smokers performed the exercise at a significantly lower peak torque than non-smokers. There was also a significant effect of time, but not a significant interaction.

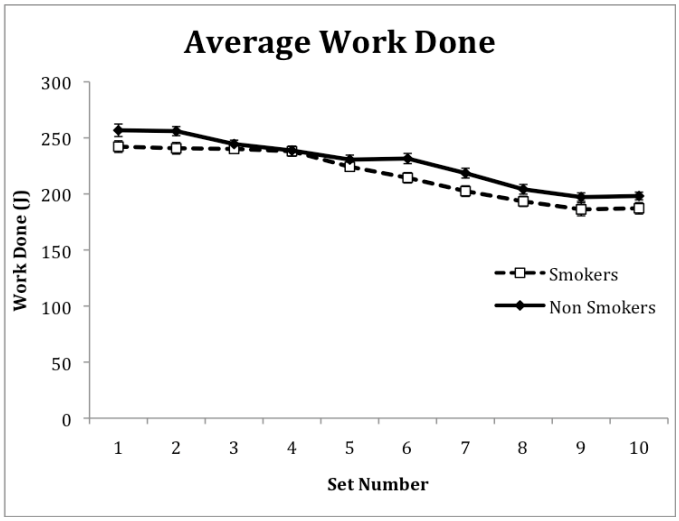


Time: p<0.01

Group: p<0.05

T*G: ns

Below is the average knee extention work done during exercise. The pattern of decrease for average work trended toward but did not reach a significant difference between smokers and non-smokers during the exercise protocol (p=0.09). There was a significant effect of time.

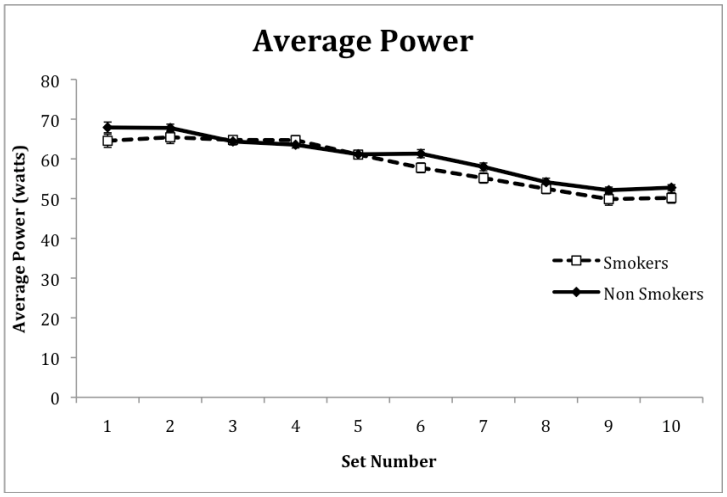


Time: p<0.01

Group: ns

T*G: ns

Below is the average knee extension power during exercise. The pattern of decrease for average power did not differ significantly between smokers and non-smokers during the exercise protocol. There was a significant effect of time.

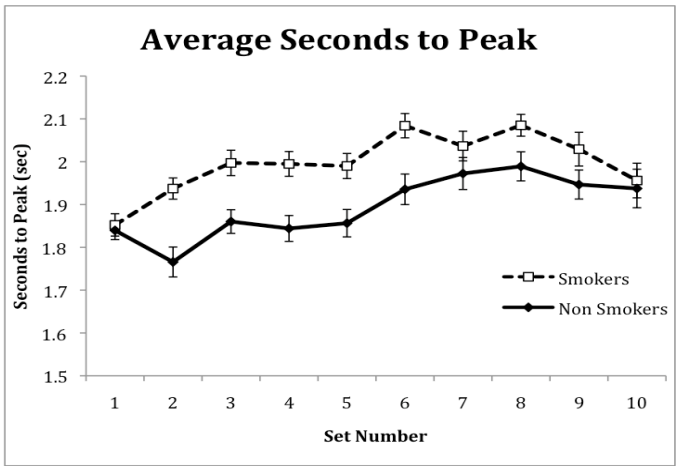


Time: p<0.01

Group: ns

T*G: ns

Below is the average knee extension seconds to peak torque during exercise. Smokers took significantly longer to reach peak torque than non-smokers. However, there was no effect of time or interaction of group and time.



Time: ns

Group: p<0.05

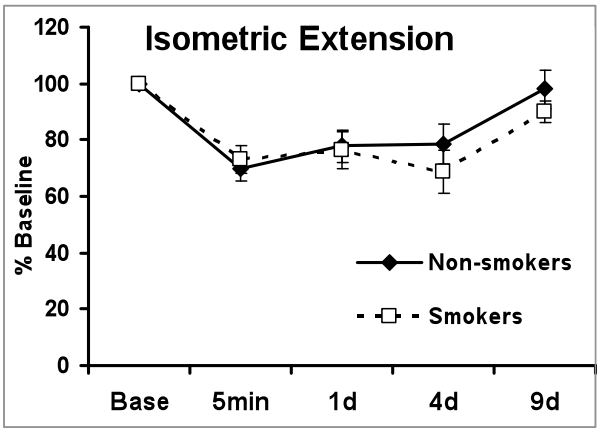
T*G: ns

Strength Alterations Over Time

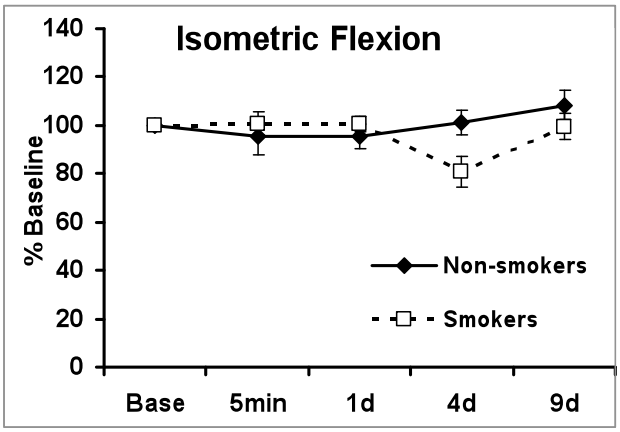
In the following three figures, the strength data are presented

Below are figures for peak isometric strength. (A) For knee extension, both groups responded similarly to exercise with significant strength loss ($p<0.05$) that persisted through 4d; at 9d both groups had nearly returned to baseline. (B) For knee flexion, there was a significant interaction between smoking status and time post-exercise. Post-hoc analysis revealed that, at 2 days post-biopsy (4 days post-exercise), smokers flexion strength decreased to 80% of baseline ($p<0.05$) while non-smokers did not exhibit strength loss. Smokers had greater strength loss in flexion than non-smokers.

(A)

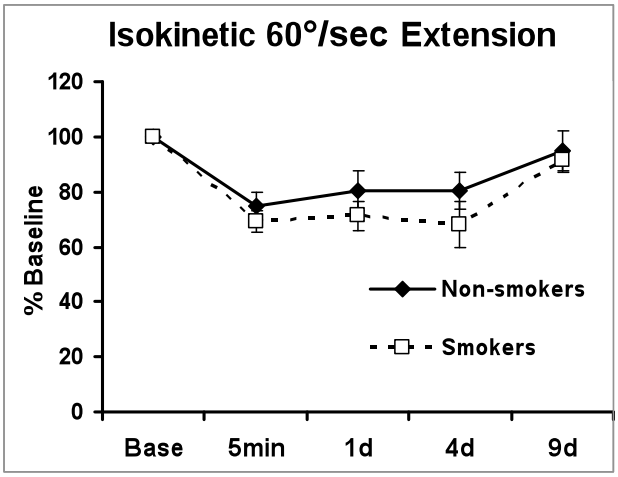


(B)

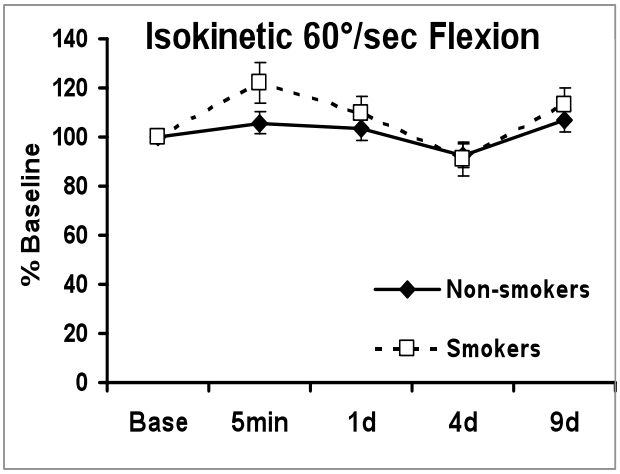


Below are figures for peak isokinetic strength (Peak Torque) at 60°/sec. (A) As expected, for knee extension there was a significant effect of time whereby all subjects lost strength initially, then recovered almost to baseline by 9d post-exercise. There was a significant difference between smokers and non-smokers, such that smokers had significantly less strength at all time points but the interaction term was not significant. (B) For knee flexion, smokers had lower baseline strength. Once baseline was applied as a covariate, there were no differences between smokers and non-smokers for flexion. Note, however, the lower strength values for smokers at 4d post-exercise.

(A)

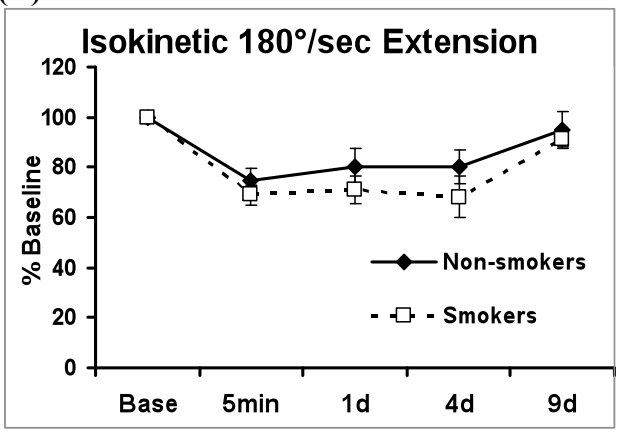


(B)



Below are figures for peak isokinetic strength at 180°/sec. (A) For extension there was a significant effect of time with a similar pattern to isokinetic at 60°/sec. There was no significant difference between smokers and non-smokers at any time point. (B) For flexion, smokers had lower baseline strength. Once baseline was used as a covariate, there were no differences between smokers and non-smokers for flexion. Note again, the drop in strength at 4d post-exercise for the smokers.

(A)



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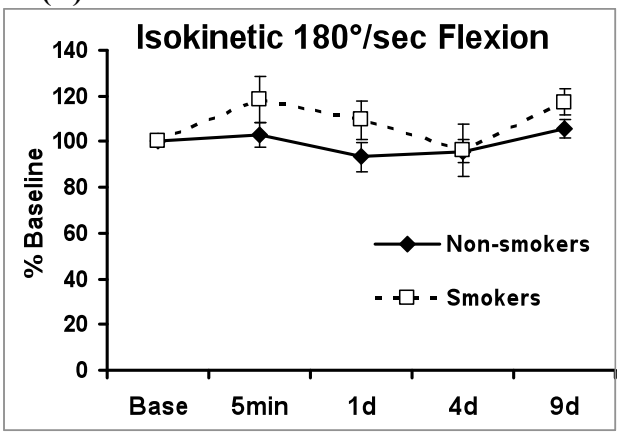
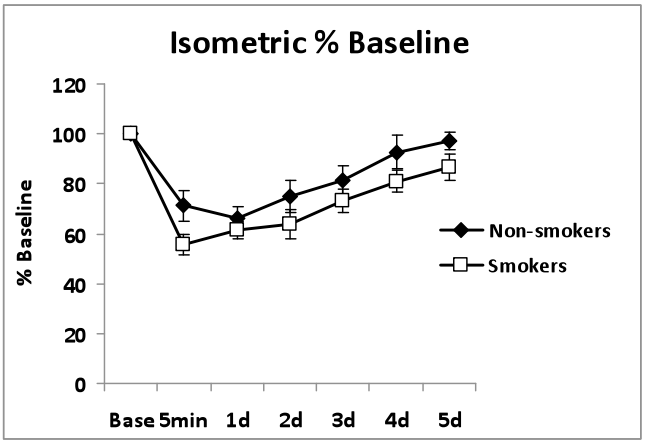
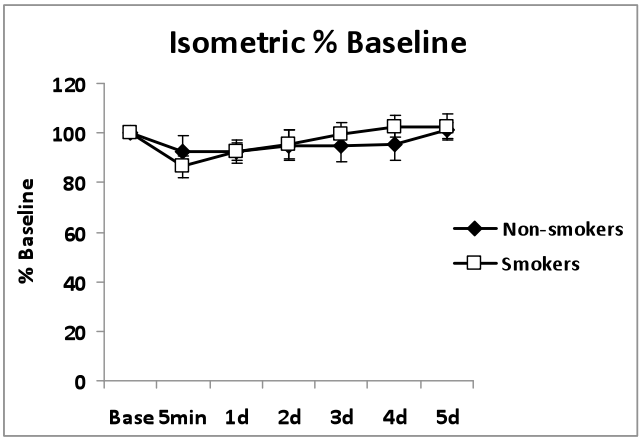


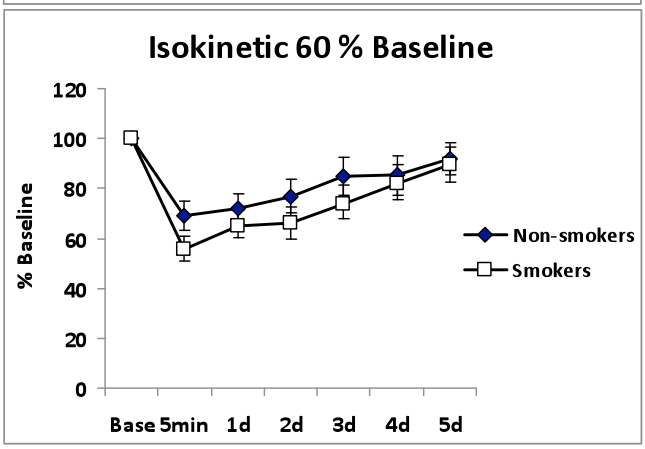
Strength Testing for an Additional Group of 10 Smokers and 10 Non-smokers

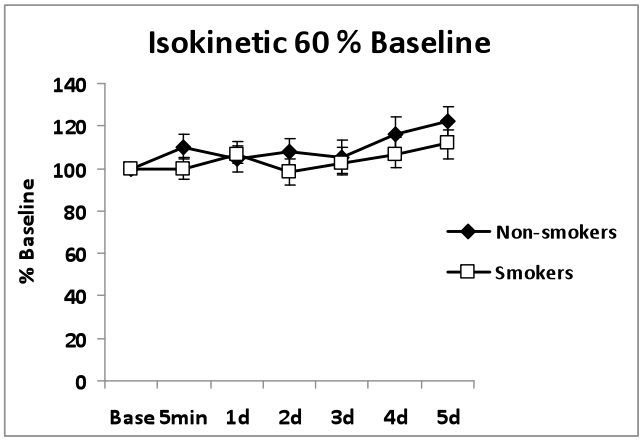
Because of the unusual exercise-induced changes in muscle strength, especially in smokers at 4d post-exercise, we wondered whether the muscle biopsy confounded the results. We were especially concerned because smokers are known to have slow healing rates. We therefore, in a separate study from this grant proposal work, recruited another group of Smokers and Non-smokers and completed identical testing except for the muscle biopsy. Below are the graphs for knee extension (left) and knee flexion (right) isometric, 60°/sec, and 180°/sec for smokers and non-smokers. For knee extension, the pattern of strength loss and restoration was similar for smokers and non-smokers and demonstrated the typical pattern of response to eccentric exercise. As we expected, knee flexion strength did not change (only the knee extensor muscles were exercised). Thus, we can conclude that the unusual strength changes at 4 d post-exercise (in the graphs above) were likely due to the biopsy and may indicate a slower healing for the smokers.

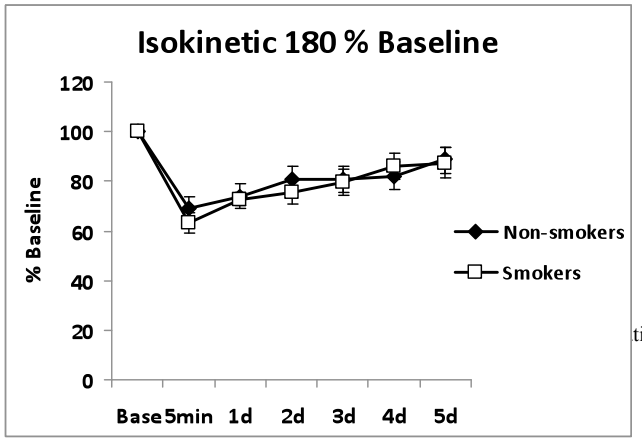
KNEE EXTENSION **KNEE FLEXION**

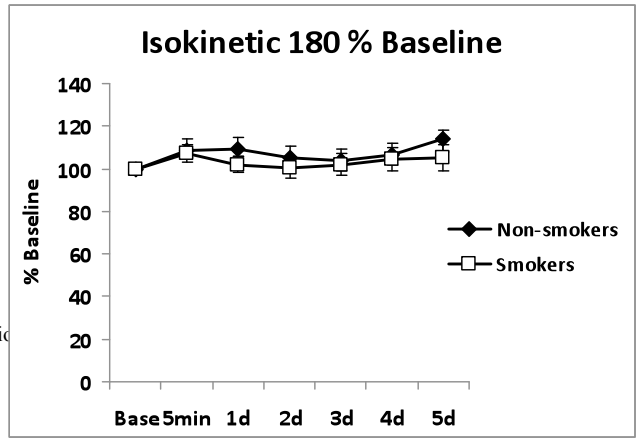






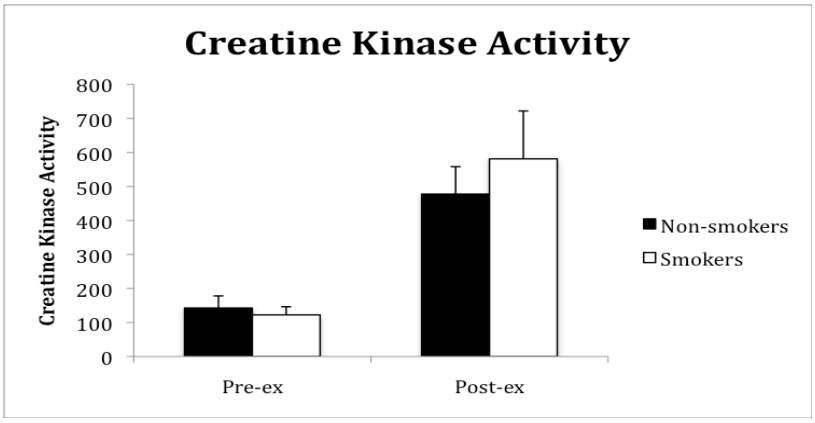






Creatine Kinase Activity Assay

Creatine kinase (CK) activity was measured at Holyoke Hospital. Blood was collected from subjects pre-exercise and 20h post-exercise.



There was a significant increased in CK post-exercise ($p<.05$) but no significant differences between smokers and non-smokers at baseline or 20h post-exercise. CK generally peaks after 20 hours; if we extrapolate the 20h results, it is possible that smokers may have greater increases in CK. We did not take blood samples beyond 20 hrs because of the potential of confounding effects of the muscle biopsies. We will follow up these results in a separate study.

PCR Arrays

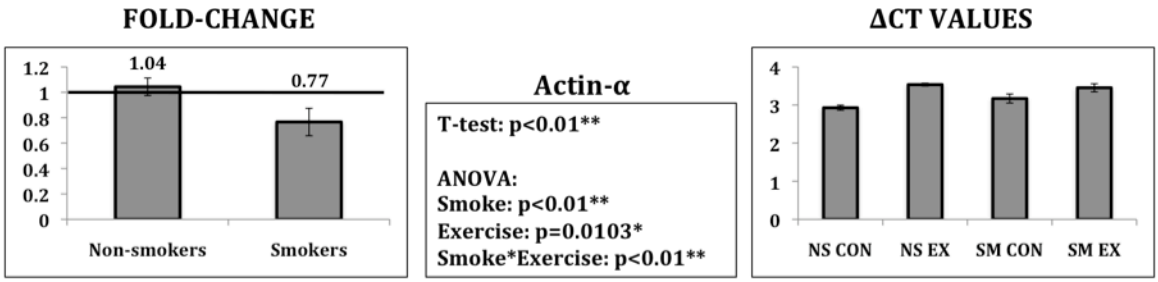
Statistics

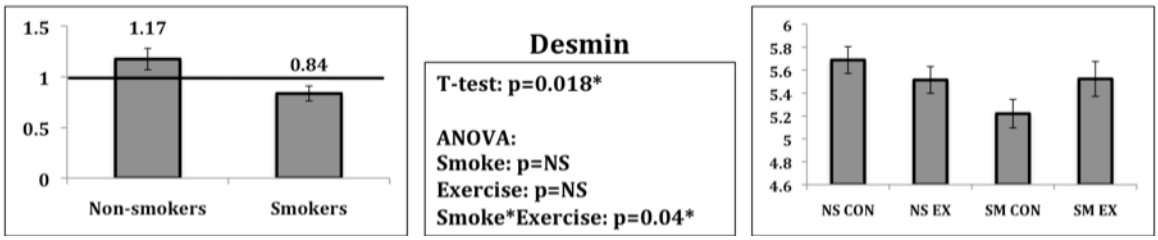
β-actin and β-2-microglobulin (B2M) were chosen as housekeeping genes because the expression of these genes remains stable after eccentric exercise and with smoking. To verify that expression was not affected by smoking or exercise, these genes were analyzed for differences between legs (control vs exercise) and smokers. Expression of the housekeeping genes was analyzed when the two expression values were averaged and each gene tested separately. In all cases, there were no significant differences in gene expression with exercise or between non-smokers and smokers. Thus, raw Ct values of all genes of interest were normalized to the average of the 2 housekeeping genes. The resulting ΔCt values were first analyzed by a one-way ANOVA using SAS statistical software for control leg differences between non-smokers and smokers. Differences in gene expression were found for 3 genes: Actin-α; the calcineurin subunit protein phosphatase 3, catalytic subunit, alpha isozyme (PPP3CA); and desmin (**see below**). For these genes, control leg was applied as a covariate in further statistical tests. All genes were then tested for significant differences using a repeated measures ANOVA and post-hoc testing with Tukey-Kramer. Data were tested for differences in expression due to exercise, smoking status, and the interaction of smoking status and exercise response. ΔCt values were also converted to fold-changes and a preliminary analysis was performed using a Student's t-test. Significance was set at $p<0.05$ for all tests.

Differences in Gene Expression for Control Leg versus Exercise

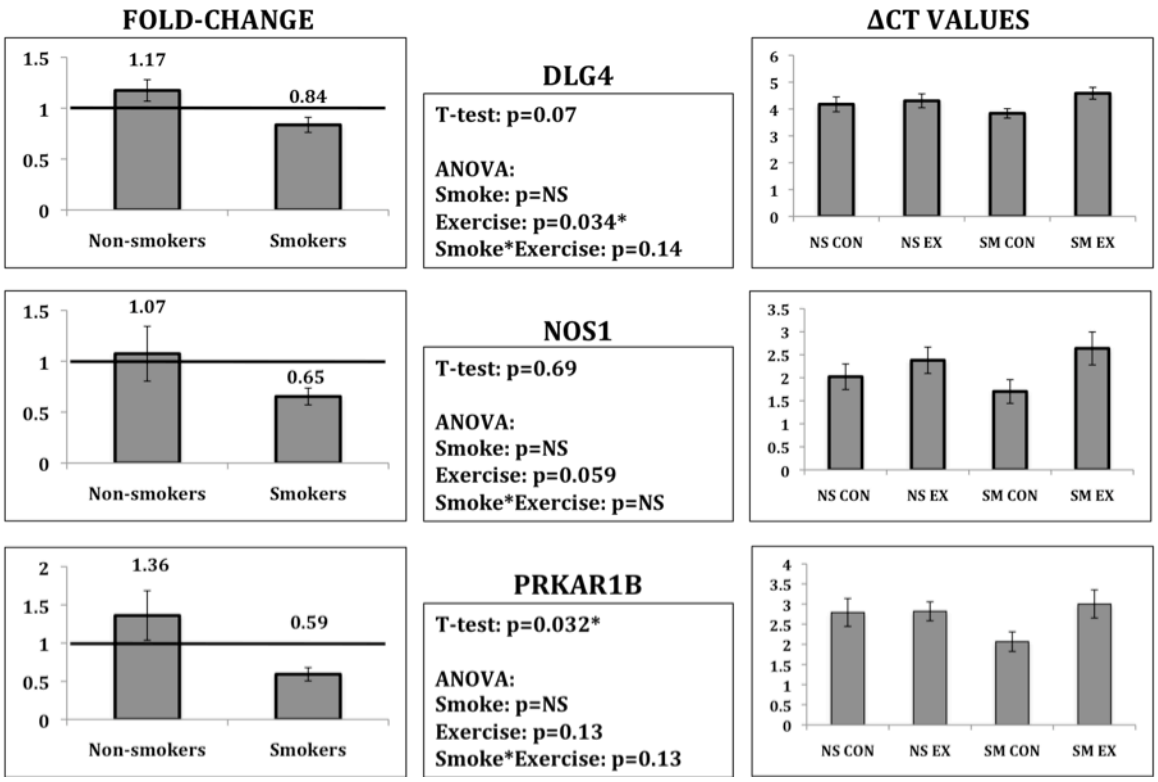
Genes that reached significance or trended toward significance are presented by functional groupings. For all of the following graphs, fold-change (of control vs exercise) is on the left and ΔCt values on the right. Please note that for ΔCt values a **larger** number is equivalent to a **lesser** amount of transcript. For fold-change, the bold line marks a 1-fold (or no change) difference. * = $p<0.05$; ** = $p<0.01$

STRUCTURAL

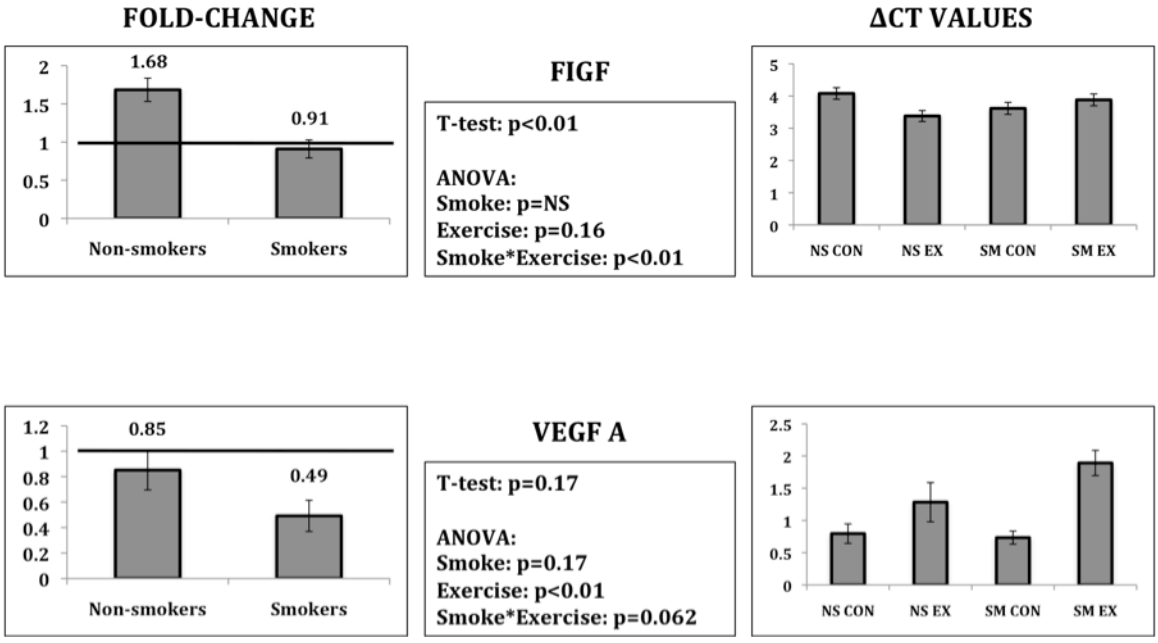




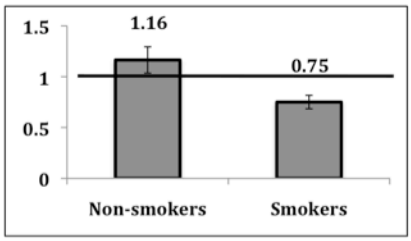
NITRIC OXIDE SIGNALING



ANGIOGENESIS/VEGF SIGNALING

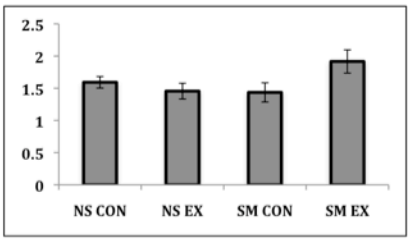


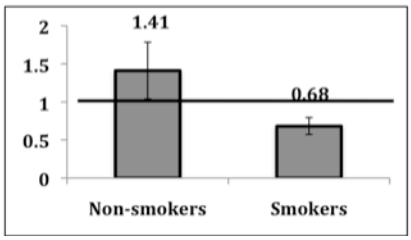
FOLD-CHANGE | **VEGF B** | **ΔCT VALUES**



T-test: p<0.01**

ANOVA:
Smoke: p=NS
Exercise: p=NS
Smoke*Exercise: p=0.032*

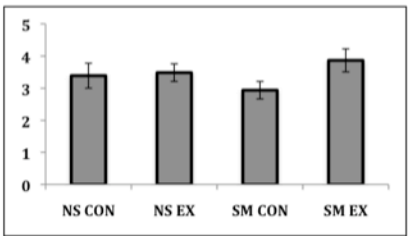




VEGF C

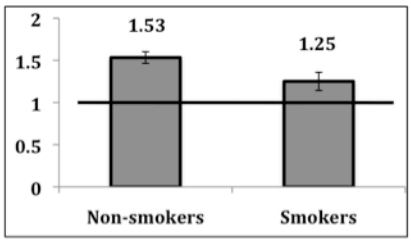
T-test: p=0.055

ANOVA:
Smoke: p=NS
Exercise: p=NS
Smoke*Exercise: p=0.18



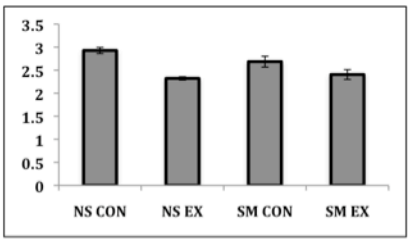
MYOGENESIS

FOLD-CHANGE | **AKT1** | **ΔCT VALUES**

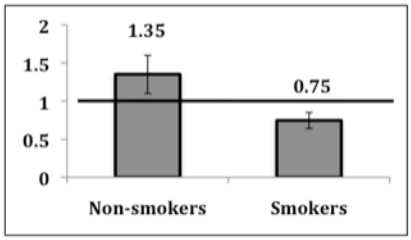


T-test: p=0.034*

ANOVA:
Smoke: p=NS
Exercise: p<0.01**
Smoke*Exercise: p=0.071

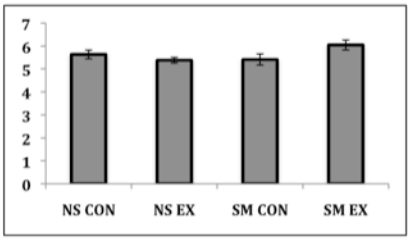


FOXO1

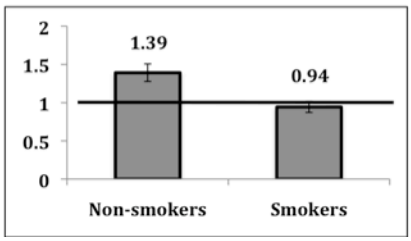


T-test: p=0.017**

ANOVA:
Smoke: p=NS
Exercise: p=NS
Smoke*Exercise: p=0.052

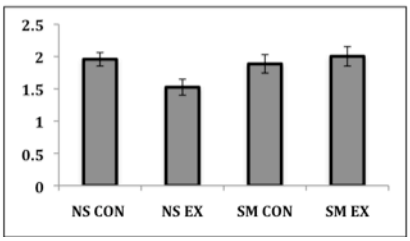


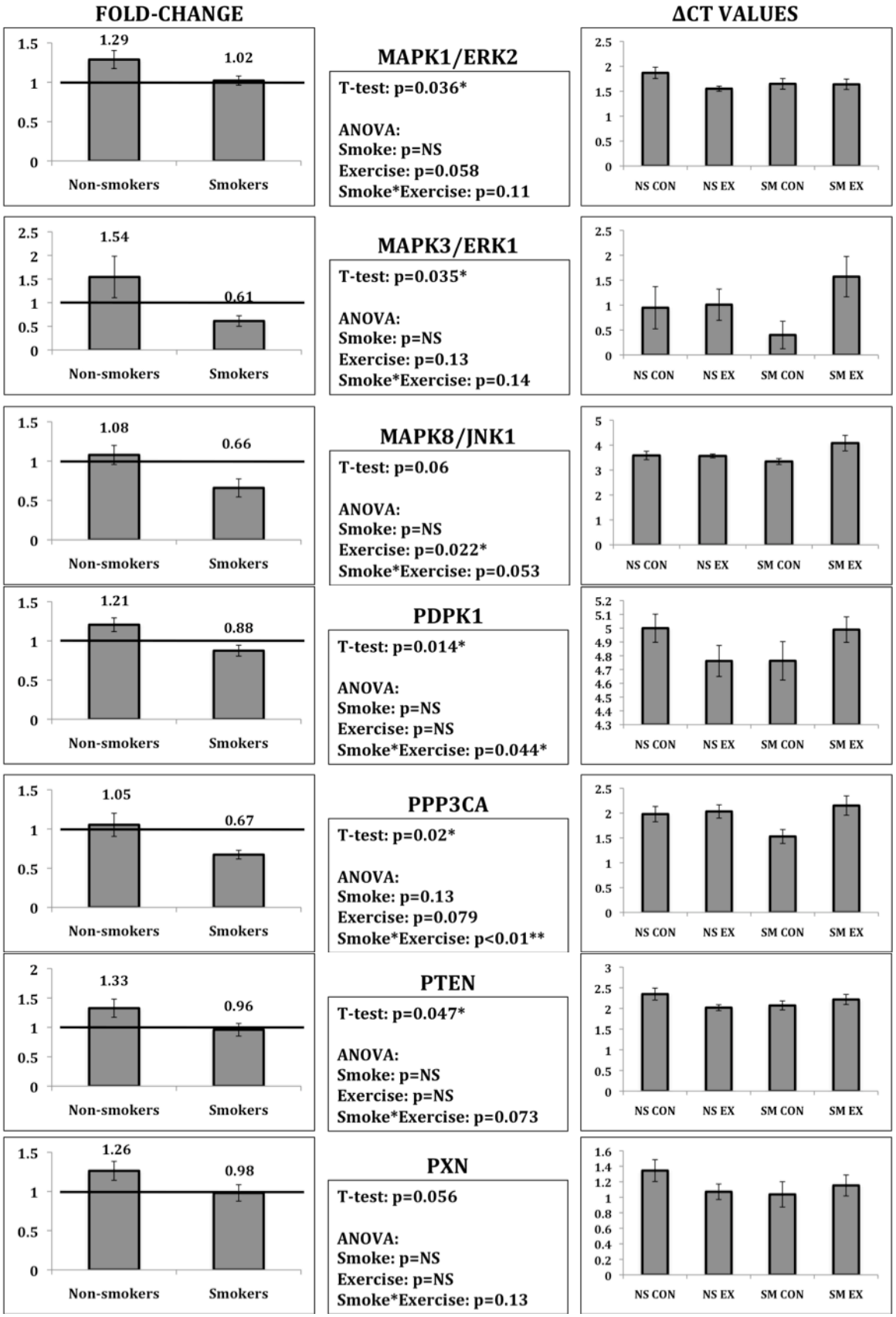
HSPA4



T-test: p=0.055

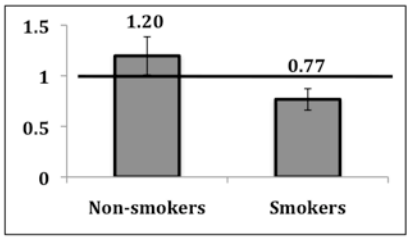
ANOVA:
Smoke: p=NS
Exercise: p=NS
Smoke*Exercise: p=0.18




FOLD-CHANGE
ΔCT VALUES
MAPK1/ERK2
T-test: p=0.036*
ANOVA:
Smoke: p=NS
Exercise: p=0.058
Smoke*Exercise: p=0.11
1.29
1.02
Non-smokers
Smokers
NS CON
NS EX
SM CON
SM EX
MAPK3/ERK1
T-test: p=0.035*
ANOVA:
Smoke: p=NS
Exercise: p=0.13
Smoke*Exercise: p=0.14
1.54
0.61
MAPK8/JNK1
T-test: p=0.06
ANOVA:
Smoke: p=NS
Exercise: p=0.022*
Smoke*Exercise: p=0.053
1.08
0.66
PDPK1
T-test: p=0.014*
ANOVA:
Smoke: p=NS
Exercise: p=NS
Smoke*Exercise: p=0.044*
1.21
0.88
PPP3CA
T-test: p=0.02*
ANOVA:
Smoke: p=0.13
Exercise: p=0.079
Smoke*Exercise: p<0.01**
1.05
0.67
PTEN
T-test: p=0.047*
ANOVA:
Smoke: p=NS
Exercise: p=NS
Smoke*Exercise: p=0.073
1.33
0.96
PXN
T-test: p=0.056
ANOVA:
Smoke: p=NS
Exercise: p=NS
Smoke*Exercise: p=0.13
1.26
0.98

INFLAMMATION

FOLD-CHANGE

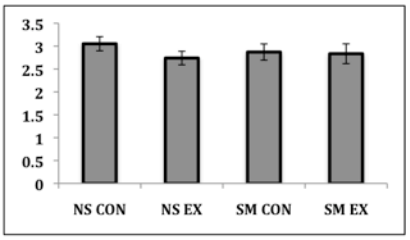


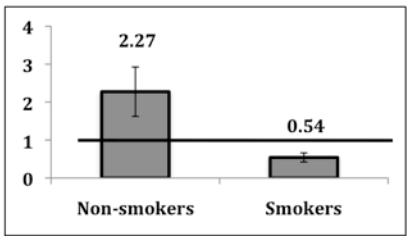
CASP9

T-test: p=0.036*

ANOVA:
Smoke: p=NS
Exercise: p=NS
Smoke*Exercise: p=0.096

ΔCT VALUES

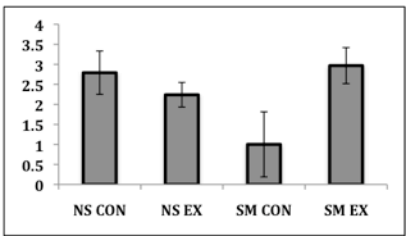




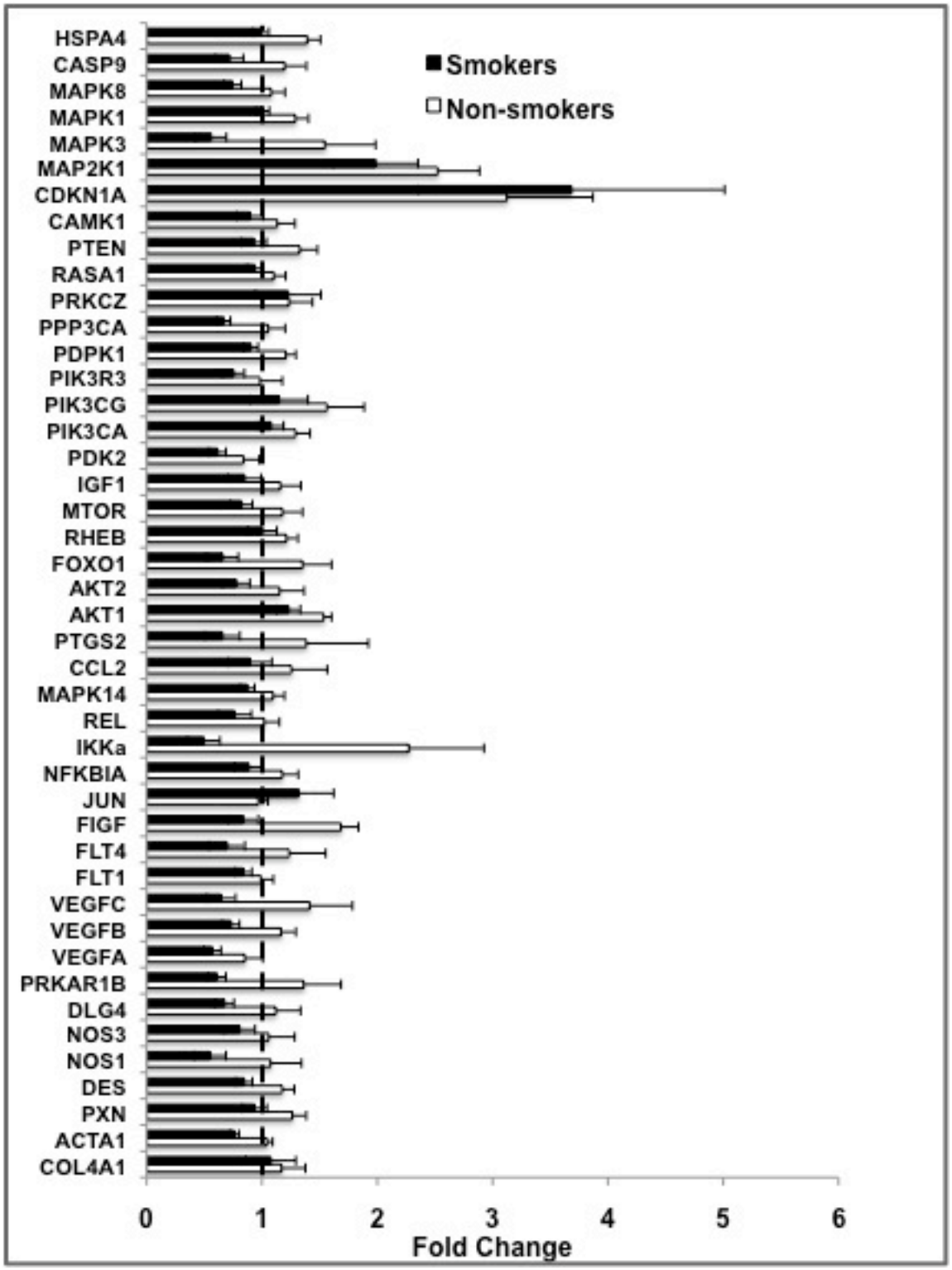
CHUK

T-test: p=0.012*

ANOVA:
Smoke: p=NS
Exercise: p=NS
Smoke*Exercise: p=0.027*



The overall profile of fold-change differences is presented below.



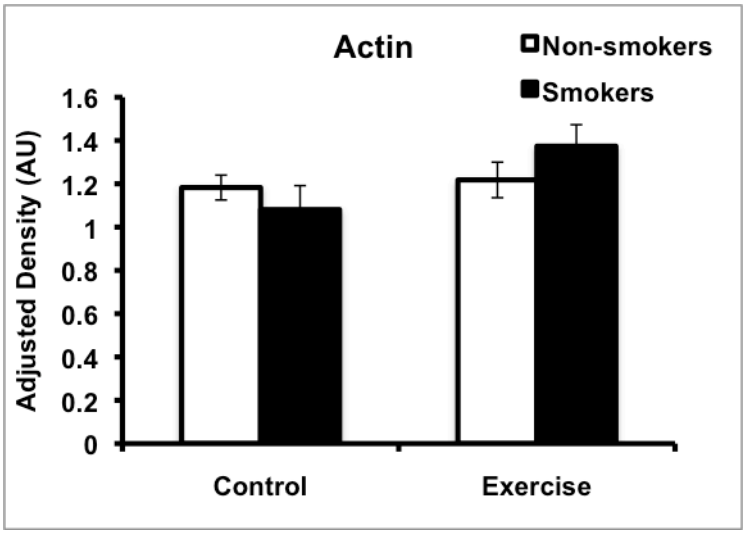
Gene expression fold-change determined using a custom-designed PCR Array. Fold-change of 1 indicates no difference between legs; above 1 indicates greater expression in the exercise leg

(upregulation), below 1 indicates less expression in the exercise leg (downregulation). Overall, non-smokers had greater expression of genes involved in muscle regeneration in the exercise leg while smokers had a decreased expression of these genes. These data indicate overall suppression of expression of genes involved in muscle regeneration in smokers, suggesting impaired recovery from injury.

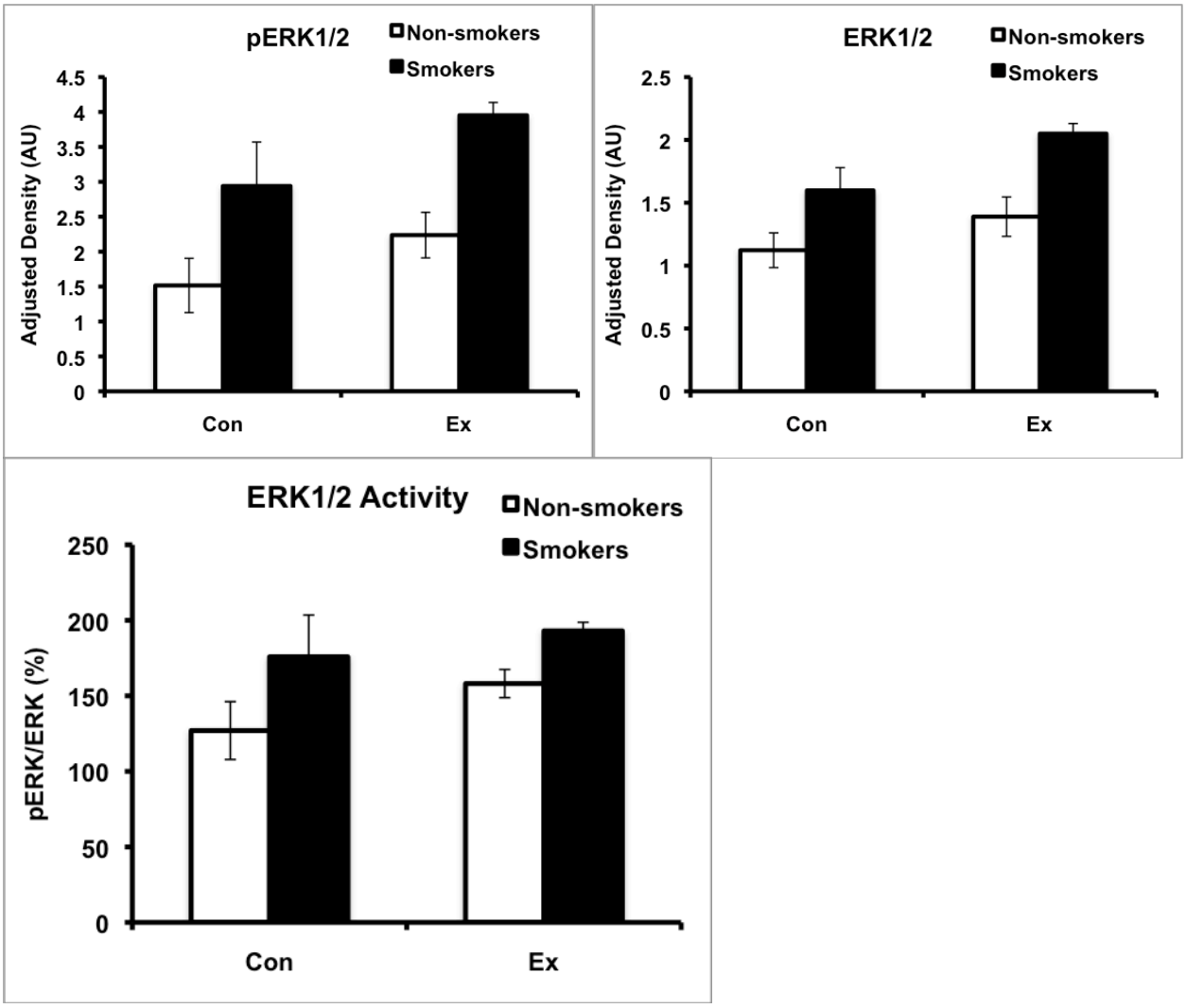
Western Blotting

Due to sample availability, a subset of samples were analyzed by western blotting for alterations in protein content. Proteins of interest were analyzed using densitometry after being normalized to a loading control (GAPDH).

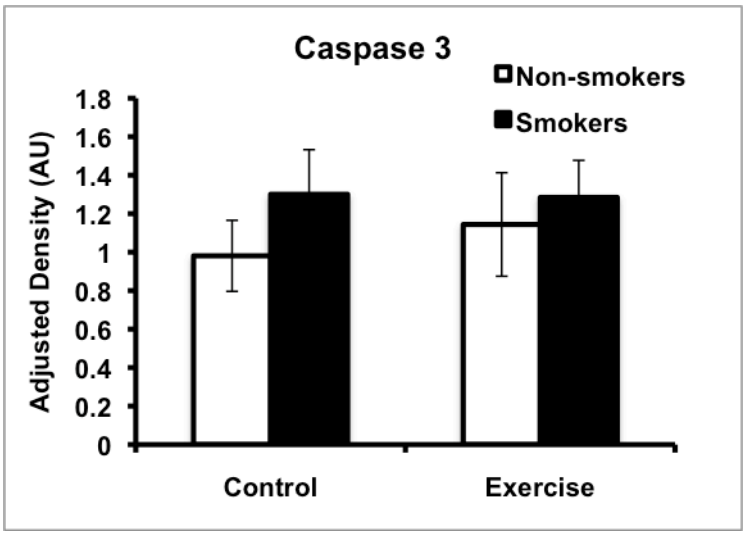
Below is a figure for α-actin. It was determined that there were no significant differences for α-actin levels between smokers and non-smokers in either leg, although there was a trend for greater protein in the exercise leg (p=0.09) that appears to be driven by the smokers.



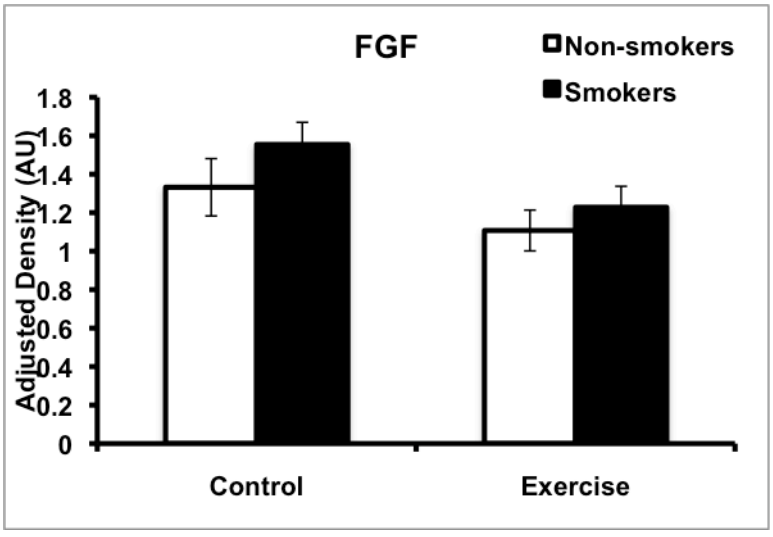
Below are figures for ERK 1/2. Analysis of total protein lysates for total ERK1/2 revealed a significant effect of group ($p<0.05$) with greater total ERK1/2 in smokers in both legs and a significant effect of exercise with increased ERK1/2 after exercise for both groups ($p<0.01$). For phosphorylated (activated) ERK1/2, there was a significant effect of time ($p<0.01$) and group, with smokers again having greater levels in both legs ($p<0.05$). When pERK1/2 was expressed as a ratio to total ERK1/2, there was a significant effect of group with smokers having greater ERK1/2 activity in both legs ($p<0.05$). However there was no significant effect of time or interaction.



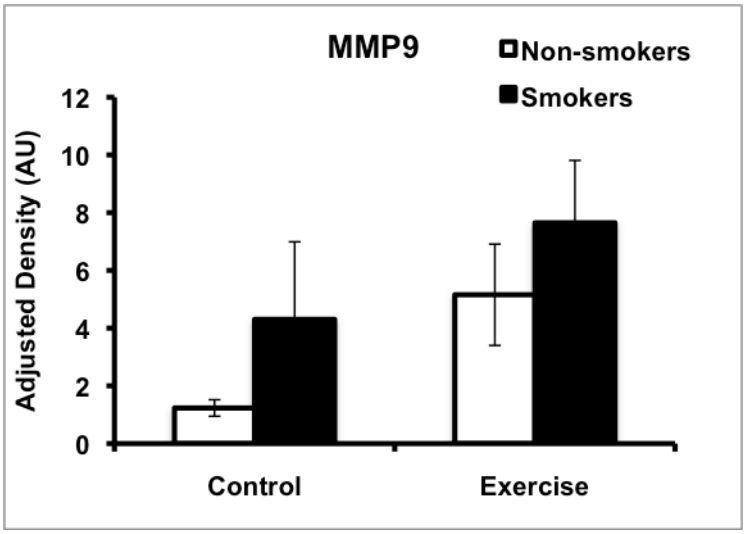
Below is a figure of caspase-3. Levels of caspase-3 were compared between legs and groups. Only faint bands were detectable, and no difference was found between groups or with exercise.



Below is a figure for fibroblast growth factor (FGF). There were no significant effects of group, time, or interaction.



Below is a figure for matrix metalloproteinase-9 (MMP9). We found no interaction of smoking and exercise or effect of exercise; however, smoking status trended toward resulting in higher levels of this protein in both legs (p=0.1).

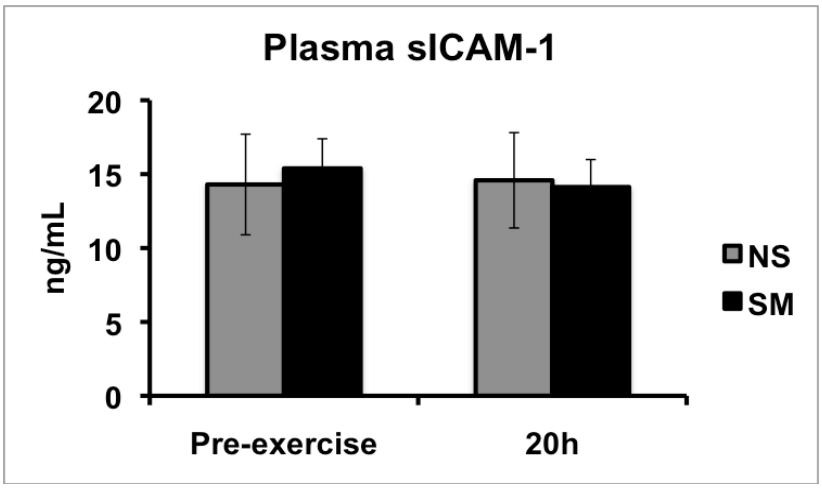


Western blotting was also done for phosphorylated AKT at thr308 and ser473, pan AKT, phosphorylated and total IKKα and β, collagen IV, desmin, and F-box only protein 6 (FbxO6). However, these are still being optimized.

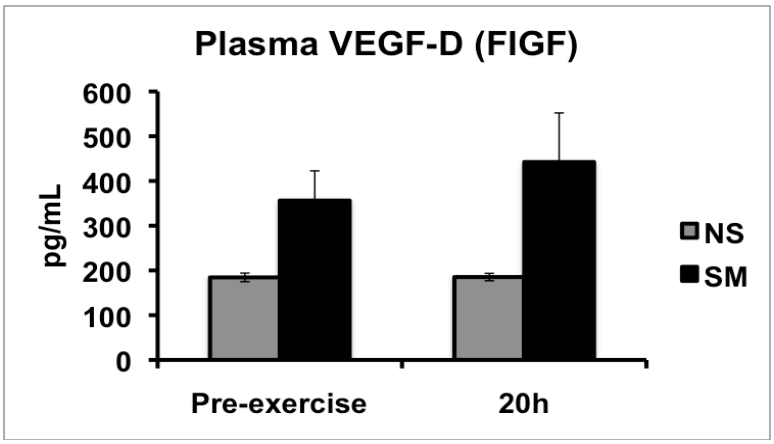
Blood Cytokine Analysis

Plasma and serum samples were analyzed for FIGF (VEGF-D), soluble ICAM-1, interleukin-6 (IL-6), C-reactive protein (CRP), interleukin-8 (IL-8), and granulocyte-colony stimulating factor (G-CSF) at baseline and 20h post-exercise. IL-6, IL-8, and G-CSF were below detectable range for most samples (results not shown). Testing was repeated using a high-sensitivity assay for IL-6; however, a more sensitive test was not available for IL-8 or G-CSF. Results for sICAM-1, FIGF, CRP, and IL-6 are displayed below.

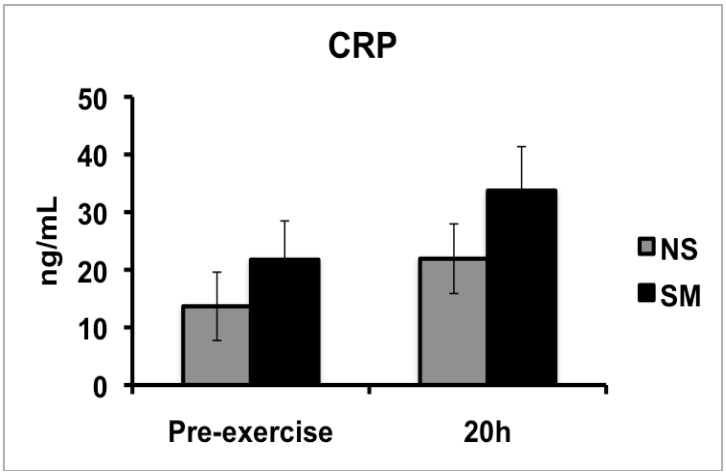
Below is a figure for sICAM-1: Smokers and non-smokers exhibited similar levels of plasma sICAM-1. Neither group had altered sICAM-1 levels in response to exercise.



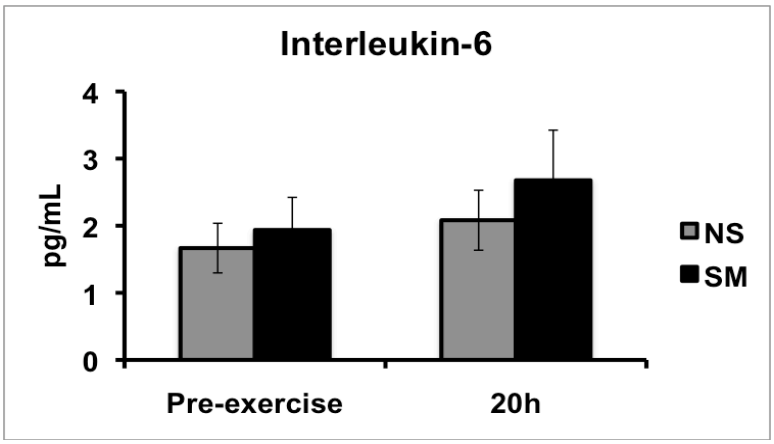
Below is a figure for FIGF (VEGF-D): There was a significant group effect, whereby smokers had higher circulating levels of FIGF than those of non-smokers ($p<0.05$). There was no effect of exercise for smokers or non-smokers and no significant interaction.



Below is a graph for CRP. There was a significant effect of exercise, whereby both groups had increased levels of circulating CRP after exercise ($p<0.05$), but no effect of group and no significant interaction.

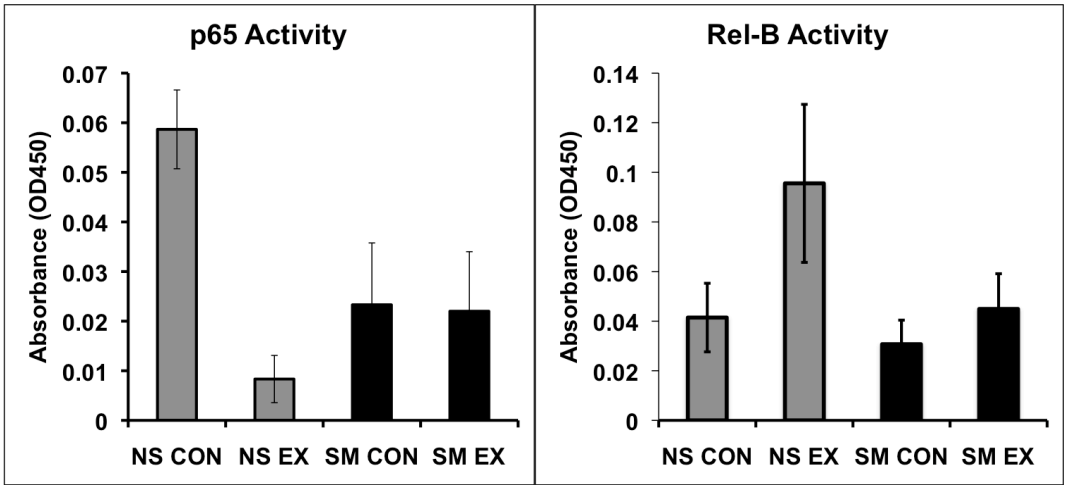


Below is a graph of IL-6. There was no difference in circulating IL-6 at baseline or after exercise between groups.



NFκB Activity

Activity of the canonical and non-canonical NFκB pathways (measured by p65 and Rel-B, respectively) was measured using DNA-binding ELISAs (Active Motif, Carlsbad CA).



In the control leg, non-smokers (NS) had greater NFκB canonical pathway activity (p65) than smokers (SM). There were no differences between the two groups for Rel-B (non-canonical pathway) activity. At 48h post-eccentric exercise, p65 activity was lower in the exercise leg (EX) than control (CON) in NS while SM (smokers) exhibited no difference. For Rel-B, NS had increased activity in EX while SM again exhibited no difference between legs.

Adverse Event Subject Data Analysis

The subject who developed a hematoma, herein referred to as the case subject, did not differ from other subjects. He was a non-smoker who did not take any medications. A table of events is presented, below. The exercise visit occurred without incident, as did the biopsy procedure. Other than a profound drop in extensor strength at 2d post-biopsy/4d post-exercise, the case subject appeared to recover strength similarly to other subjects. He reported progressive improvements in soreness and stiffness each day. At 4d post-biopsy/6d post-exercise, the case subject spent 5-6h standing in the course of his work. The following evening, he developed transient cramping and pain in the exercise leg, which resolved when the subject lay down. The next evening (6d post-biopsy/8d post-exercise), the case subject reported rapidly increasing muscle cramping, pain, and swelling in the exercise leg. He went to the emergency department at a local hospital and was admitted. Initial laboratory results (Table 2) showed a slightly elevated white blood cell count, elevated neutrophils, and a slight elevation of C-reactive protein (0.8 mg/dL); low red blood cell count, hemoglobin, and hematocrit; and normal blood clotting parameters (prothrombin time, INR, and activated partial thromboplastic time). Blood creatine kinase (CK) activity upon admittance was elevated at 5,630 U/L. CK MB levels were within normal ranges. All measures for urinalysis were normal including lack of hematuria. An orthopedic surgeon examined the subject and differential diagnosis focused on compartment syndrome, rhabdomyolysis, infection, and hematoma. Compartment syndrome was eliminated due to the lack of excruciating pain, absence of severe tension, and the subject's leg could be flexed passively without discomfort to 30-45 degrees of flexion. Lack of hematuria coupled with relatively modest CK elevation excluded diagnosis of clinically relevant rhabdomyolysis (i.e.

danger of renal failure). X-ray of the thigh showed soft tissue swelling but normal bone mineralization and shape; no air or gas was present in the soft tissue. These findings excluded diagnoses of fracture or anaerobic infection. To assess the soft tissue swelling, the orthopedic surgeon measured the subject's leg circumference with a tape measure. The non-dominant leg measured 44.45 cm in circumference at 15.24 cm above the superior pole of the patella as compared to 41.91 cm in the dominant, non-exercised leg. The final diagnosis was presumed to be intramuscular hematoma.

After 3d of local care, including ice and pain medication, the subject was released from the hospital. With 2 months of physical therapy, he returned to complete normal activity, including recreational running.

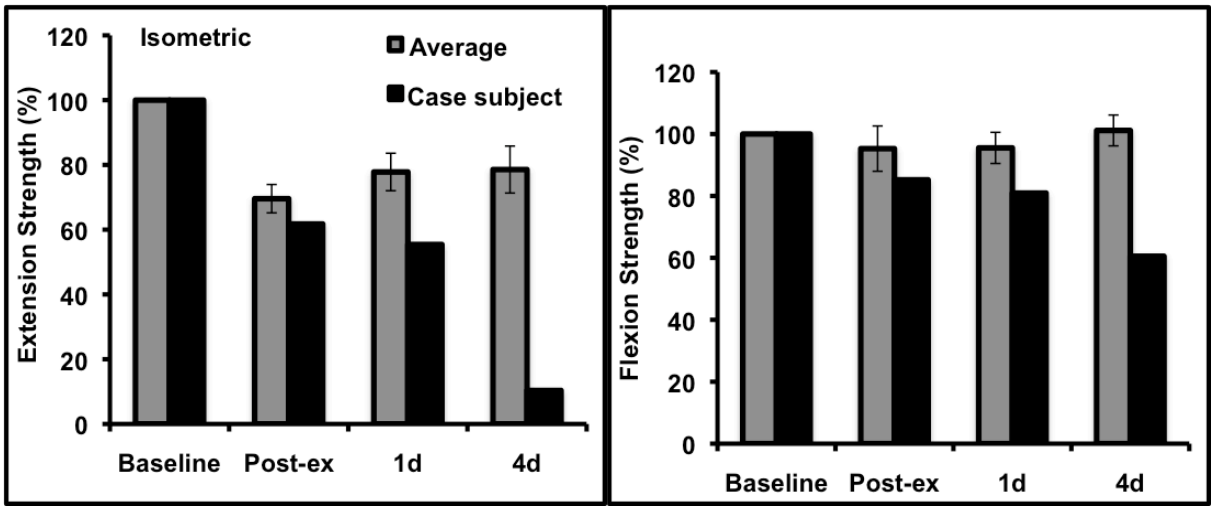
Table 2: Timeline of events

Days post-exercise	Days post-biopsy	Comments
0	0	Exercise procedure normal
2	Biopsy	Biopsy procedure normal
3	1	Reported no undue pain, no report of unusual weakness
4	2	Significantly lower strength than 1d post-exercise and compared to other subjects; reported no undue pain, no report of unusual weakness
5	3	Symptoms abating
6	4	Subject spent 5-6h standing; symptoms abating
7	5	Evening: transient cramping in exercise leg, resolved when lying down
8	6	Evening: increasingly intense cramping and pain in exercised leg; went to emergency room at hospital and admitted

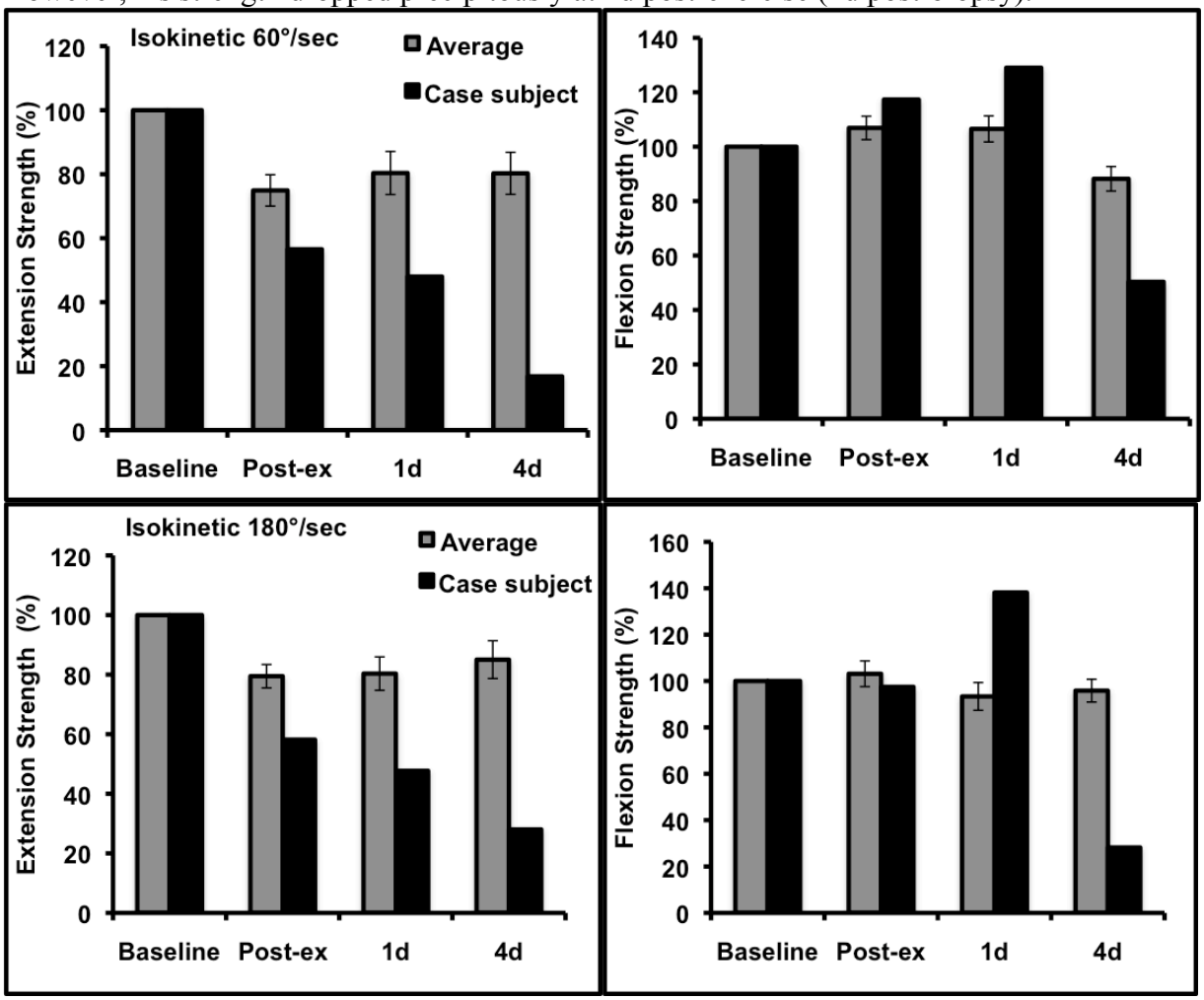
Strength

In the following three figures, the strength data for the case subject as compared to the average of all other non-smoking subjects are presented (isometric and isokinetic at 60°/sec and 180°/sec).

Below are the figures for isometric strength. For extension (at left), the case subject, shown in the black bars, continued to lose strength while other non-smoking subjects returned toward baseline. At 4d post-exercise (2d post-biopsy) the case subject had dropped to 10% of his baseline strength. For flexion (at right), the case subject remained below the other non-smokers and was at approximately 60% of baseline strength at 4d post-exercise.



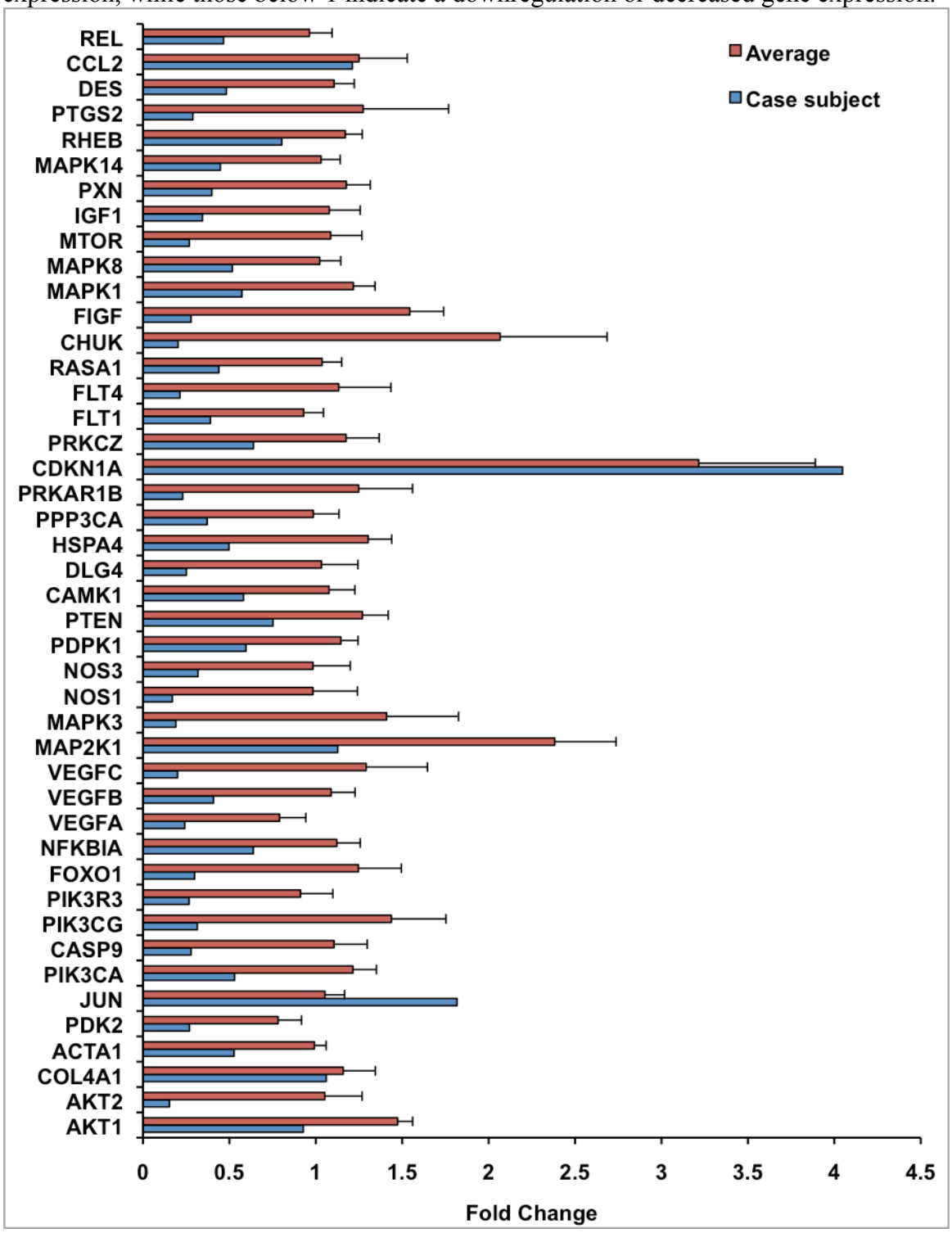
Below are the figures for isokinetic strength at 60°/second (top) and 180°/second (bottom). Similar to the isometric strength data for extension (at left), the case subject continued to lose strength while other non-smoking subjects returned toward baseline. For flexion (at right), the case subject remained near baseline immediately post-exercise and increased strength at 1d. However, his strength dropped precipitously at 4d post-exercise (2d post-biopsy).



PCR Array

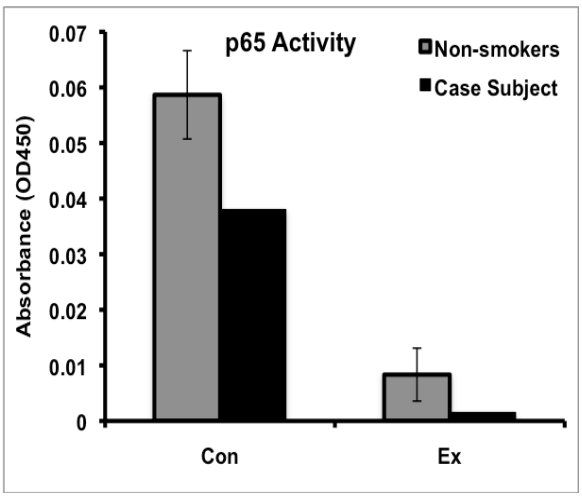
A biopsy sample from the case subject was analyzed using PCR arrays and compared with array data collected from the other non-smoking subjects. The profile of gene expression changes is presented below. Genes did not differ in the control (non-exercise) leg as compared to other non-smokers. However, while non-smoking subjects tended toward increased or no change in gene expression of the 44 genes tested that relate to muscle regeneration, the case subject responded to the same exercise with a downregulation of all but 6 genes. AKT1, Collagen 4 (COL4A1), MAP2K1, and CCL2 did not differ between control and exercise leg in the case subject while there was an upregulation of these genes in the remaining non-smokers. JUN and CDKN1A expression was increased in the case subject above that of the other non-smokers, although not profoundly so for CDKN1A. **These data are particularly compelling as they show that the case subject was experiencing altered gene expression at 2d post-exercise, well before he exhibited symptoms**. This may indicate that the subject responded to the exercise with impaired

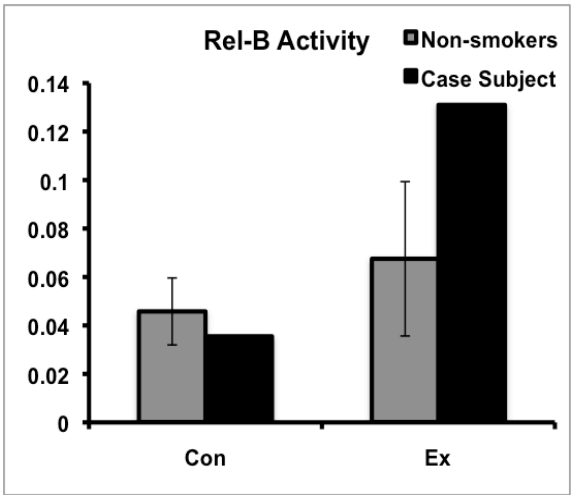
regenerative mechanisms, potentially prolonging his recovery time and making him more susceptible to further injury and hematoma. Below are the gene expression changes with exercise in the case subject (blue) and all other non-smokers (red). A value of 1-fold indicates no alteration in gene expression. Values above 1 represent an upregulation or increased gene expression, while those below 1 indicate a downregulation or decreased gene expression.



NFκB Activity

Activity of p65 and Rel-B were measured as previously discussed for smokers and non-smokers. Data from the non-smokers were compared to the adverse event (case) subject. Graphs are presented below. The case subject showed a lower baseline for p65 and responded a greater degree, for both p65 and Rel-B, to the exercise.





Presentations and Publications

Data for isometric and isokinetic strength over time were presented at the annual fall meeting of the New England chapter of the American College of Sports Medicine in November, 2010 in Providence, RI (**Appendix C**). The significant adverse event was accepted as an abstract for presentation at the national meeting of the American College of Sports Medicine in May, 2011 in Denver, CO (**Appendix D**). The significant adverse event was also prepared for publication as a case study and submitted to *Medicine and Science in Sports and Exercise* in November, 2010 (**Appendix E**). This was not accepted in its present form primarily because of lack of confirming information that a hematoma did occur (e.g. ultrasound analysis). However, we will add the molecular data to the manuscript and submit for publication in future because the subject who had the AE had a very different molecular response to the exercise. Data was presented at the annual fall meeting of the New England chapter of the American College of Sports Medicine on November 3rd, 2011 (**Appendix F**) and an abstract submitted for presentation at the national meeting of the American College of Sports Medicine in May-June, 2011 in San Francisco, CA (draft attached in **Appendix G**).

SUMMARY OF MAJOR FINDINGS

- Analysis of isometric and isokinetic strength showed that smokers and non-smokers had similar strength loss and recovery patterns in response to the eccentric exercise.
- Smokers showed an unusual trend for a lower strength at 4d post-exercise. This is likely due to the confounding effects of the biopsy and slower healing rate for smokers.
- PCR arrays revealed differences between smokers and non-smokers for genes relating to muscle regeneration, angiogenesis, nitric oxide signaling, inflammation, and structure.
- Analysis of the hematoma case subject revealed alterations in strength and RNA expression days before the subject developed symptoms typical of hematoma. RNA expression of genes relating to muscle regeneration was primarily repressed. This could imply that muscle repair may have been compromised in this subject, leading to a greater susceptibility for developing a hematoma with increased activity.
- NFκB pathways in muscle samples were altered in non-smokers, with decreased canonical pathway (p65) activity and increased non-canonical (Rel-B) activity. Conversely, there were no differences between control and exercise leg in smokers for NFκB activity. Further, p65 activity was lower in the control leg of smokers than non-smokers. This may indicate altered NFκB signaling in the muscle of smokers, leading to impaired muscle healing after injury.
- ELISA analysis of circulating factors revealed higher levels of FIGF (VEGF-D) in smokers and increased levels of CRP after exercise in both groups. No differences were found for sICAM-1 and IL-6. Western blotting revealed significantly higher levels of phosphorylated and total ERK1/2 in smokers. MMP9 levels were higher in smokers, which trended toward significance in both legs.

CONCLUSION

The analysis of the skeletal muscle using PCR arrays provided an overall profile of expression changes for many key pathways in muscle function that differ between smokers and non-smokers in response to strenuous eccentric exercise. We found that smokers had attenuated expression of genes (mRNA) at 48h post-exercise while non-smokers generally increased expression of the same genes. These genes were clustered into functional groups (structural proteins, nitric oxide signaling, angiogenesis, inflammation, and myogenesis), most of which have been shown to be involved in muscle regeneration in some capacity. We identified that, not only was expression of α-actin and desmin lower in the control leg of smokers than non-smokers, but also this expression was attenuated further with exercise in smokers. We also found that expression changes of the genes FIGF (VEGF-D) and VEGF-B was suppressed in smokers with exercise. These genes are involved in angiogenesis and vascularization, processes important to muscle regeneration. Additional genes significantly lower with exercise in smokers were PDPK1 (PDK1) and PPP3CA, a subunit of calcineurin. PDPK1 is an upstream regulator of the AKT protein synthesis pathway that is activated during muscle regeneration; calcineurin has been implicated in muscle differentiation and regeneration.

Of the 44 genes measured with the array, we found that CHUK (IKKα) showed the greatest differences between smokers and non-smokers. This led us to examine potential differences in NFκB since IKK is an upstream mediator of NFκB activity. We found that NFκB activity, which was altered in non-smokers, remained unchanged in smokers in response to exercise. NFκB is a key player in the acute inflammatory response to exercise.

Circulating blood levels of FIGF (VEGF-D), one of the genes found to be suppressed in smokers using the PCR array, was measured at 20h post-exercise. We found that smokers had higher circulating levels both at baseline and post-exercise. Expression of the FIGF gene was suppressed in muscle approximately 1d later than the 20h blood draw, which may indicate that circulating levels of FIGF may decrease at a later time point after exercise. Circulating CRP, a measure of systemic inflammation, was also found to be higher at both pre-exercise and 20h after in smokers. However, levels changed similarly to non-smokers with exercise. We investigated circulating levels of sICAM-1, a chemokine, and IL-6, a cytokine, both of which are involved in inflammatory signaling after exercise. Further, IL-6 can stimulate the NFκB pathway. We did not find significant changes with exercise or differences between smokers and non-smokers for either factor; however, they may have been altered near the site of muscle injury, and therefore systemic levels could have appeared similar due to dilution throughout the circulation.

Because expression of many genes involved in muscle regeneration was differentially regulated in smokers and non-smokers in response to exercise, we decided to examine the protein products of several of these genes. Phosphorylated and total ERK1/2 levels were higher in smokers than non-smokers, when presented as a ratio, did not change with exercise while non-smokers had increased ERK1/2 activation. MMP9, which contributes to the breakdown of muscle after exercise and is stimulated by ERK1/2, was also higher in smokers than non-smokers, and did not change significantly with exercise.

A number of circulating factors and skeletal muscle genes and molecular pathways were found to be altered with smoking and exercise, all of which affect muscle regeneration. The NFκB and ERK1/2 pathways in particular serve several roles in regeneration including myogenesis and

inflammation. Dysregulation of these pathways may result in impaired muscle regeneration after a damaging exercise that, over time, could lead to muscle that is more susceptible to injury. These data offer important insight into a mechanism that could explain the higher risk for musculoskeletal injury in smokers.

Adverse Event

One subject (non-smoker) developed an unusual, late-appearing hematoma at 6d post-biopsy (8d post-exercise). We found that compared with other non-smokers, his strength was severely impaired at 2d post-biopsy. Furthermore, gene expression was suppressed in the exercise leg for almost all of the 44 genes tested when compared to other non-smokers. Because these genes are involved in muscle regeneration, we hypothesize that this individual may have had suppression of genes that would normally assist a subject to recover from the exercise and the biopsy and that this suppression led to increased risk for developing a hematoma. These novel data may provide mechanisms to explain why some subjects develop complications after injury and are particularly compelling given that this subject's gene expression differed profoundly from other non-smokers at the time of the biopsy.

Future Analyses

Continuing analyses will focus on other proteins in the NFκB , ERK1/2, and AKT pathways. In particular, measuring IKK activity is a next important step in exploring this area of research. Visualizing the localization of these differences is also critical to understanding how these pathways may be influencing muscle regeneration. Therefore, immunohistochemistry will follow shortly, specifically focusing on whether these pathways are activated in muscle or other cell types, such as pericytes and inflammatory cells. It will also be important to visualize molecular changes to determine if there are disruptions to the extracellular matrix and other structural proteins that could indicate differences in the damage/remodeling process. All antibodies and chemicals are in hand to complete this work. When completed, we believe that our data will provide a model showing that muscle regeneration is impaired in smokers and that this impairment is due to the interaction of multiple pathways (including NFκB , ERK1/2, and AKT). Because NFκB and ERK1/2 are important players in inflammation, we believe that differences in inflammation may be important in explaining the effects of smoking on muscle repair and risk for injury. Our future studies will therefore include additional inflammatory cytokines as well as inflammatory cells that may participate in muscle regeneration.

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The Effect of Smoking on Muscle Adaptation to Exercise Stress

First Name: _____ MI: ___ Last Name: _____

Date of Birth: __ __ / __ __ __ / __ __ __ __ Age: _____
Day Month Year

Sex: ☐ Male ☐ Female

<u>Contact Information:</u>

Mailing Address: _____

City: _____ State: _____ Zip: _____

Phone Numbers: _____ (cell)

_____ (work)

_____ (home)

E-mail Address: _____

Primary Care Physician: _____

Office phone/fax: _____

Subject Code Number: ____ ____ Subject Initials: ____ ____ ____

SUBJECT NUMBER __ __ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 0

AGE: ____

RACE:
Caucasian ☐ Native Hawaiian or Pacific Islander ☐
Black ☐
Hispanic ☐ America/Alaska Native ☐
Asian ☐ Other ☐

Dominant Side (leg): ☐ Right ☐ Left

Current smoker? ☐ Yes ☐ No

IF YES: How many cigarettes does subject smoke per day, and how long has subject smoked that amount? _____

If ≤ ½ pack per day or has been smoking < 5 years, subject cannot continue study

IF NO: Has subject ever been a habitual smoker? ☐ Yes ☐ No

If YES, subject cannot continue study

INCLUSION CRITERIA

	YES	NO
Are responses to telephone screen still accurate?		
Is subject between 18 and 35 years of age?		
Is subject willing to comply with the study conditions?		
Is subject willing to refrain from any strenuous or new physical activities while participating in this study?		
Is subject willing to refrain from taking any anti-inflammatory drugs (i.e. NSAIDS such as Ibuprofen or aspirin) or any aspirin-containing drugs such as Alka-Seltzer, Pepto-Bismol, or certain decongestants (i.e. Dristan) for the course of the study unless otherwise instructed by study staff or physician?		
Does the subject habitually take no more than 2 alcoholic drinks per day?		
Does subject fit criteria of either smoker or non-smoker?		
Does subject understand the study and give written informed consent?		

ALL ANSWERS MUST BE "YES" FOR SUBJECT TO CONTINUE WITH THE STUDY

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 0 Continued

EXCLUSION CRITERIA

	YES	NO
Does subject have an occupation requiring heavy weight lifting/lowering or have participated in weight training activity of the lower body within the past 6 months that may influence the response to study exercise as determined by a scale of known intensities (MET scale) for occupation, recreational, and activities of daily living?		
Has subject had orthopedic surgery in the leg (unless cleared by a physician) or have a skeletal, muscular or neuromuscular dysfunction?		
Has subject participated in a muscle soreness trial within the previous 6 months using the legs?		
Is subject likely to have problems successfully completing the study exercise requirements?		
Is subject using and/or has used any corticosteroids within the past 8 weeks including topical preparations? (anti-inflammatory drugs commonly used to treat allergic reactions, skins irritations, asthma, and some autoimmune disorders)		
Is subject currently taking any medication that would interfere with the study results such as medications for diabetes?		
Is subject taking any therapeutic dietary supplements (other than a vitamin and mineral supplement with $\leq$100% of the RDA) such as high protein supplements designed to increase muscle mass or to lose weight or stimulant containing products such as those containing ephedra?		
Does the subject regularly consume any narcotic preparation (e.g. codeine) or illicit drugs (such as marijuana, etc.) or has within the previous 7 days?		
Does subject have any conditions or diseases including: cardiovascular, pulmonary, metabolic (diabetes), or chronic diseases?		

ALL ANSWERS MUST BE "NO" FOR SUBJECT TO CONTINUE WITH THE STUDY

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 0 Continued

Informed Consent form administered and discussed? ☐ Yes ☐ No

UN-signed copy of consent given to subject: ☐ Yes ☐ No

Subject instructed to return before signing consent? ☐ Yes ☐ No

Have any adverse events occurred during *this* study visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

PROGRESS NOTES:

_____

_____

NEXT STUDY APPOINTMENT

Date of Next Visit: __ __ / __ __ __ / __ __ __ __ __ __ __:__ __
Day Month Year (24-hour clock)

Note: Must be within 2 weeks of Visit 0

Was subject instructed to refrain from consuming any medications that have anti-inflammatory properties within 48 hours of Visit 1 (may include cold/flu medications, NSAIDS), caffeine within 12 hours of Visit 1, and alcohol within 24 hours of Visit 1?
☐ Yes ☐ No

Was subject instructed to not eat breakfast (fast at least 8 hours) before Visit 1?
☐ Yes ☐ No

Visit Data Collected By:

_____ _____ _____
Printed Name Signature Date

_____ _____ _____
Printed Name Signature Date

END OF VISIT

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 1

Does subject continue to meet all Inclusion/Exclusion criteria? ☐ Yes ☐ No
If no, describe _____

INFORMED CONSENT FORM SIGNED __ __ / __ __ __ / __ __ __ __
Day Month Year

Written consent obtained by: _____

Signed copy of consent given to subject: ☐ Yes ☐ No

HEALTH HISTORY QUESTIONNAIRE COMPLETED __ __ / __ __ __ / __ __ __ __
Day Month Year

PAR-Q COMPLETED __ __ / __ __ __ / __ __ __ __
Day Month Year

Has subject had a cold in the past 24 hours?
☐ Yes (must reschedule) ☐ No (continue)

Has subject consumed any non-steroidal anti-inflammatory medicine or aspirin containing medicine in the past 24 hours?
☐ Yes (must reschedule) ☐ No (continue)

Has subject consumed any alcohol in the past 24 hours?
☐ Yes (must reschedule) ☐ No (continue)

Has subject consumed any caffeine in the past 12 hours?
☐ Yes (must reschedule) ☐ No (continue)

Has the subject fasted? (no food/beverage besides water for 8-12 hours)
☐ Yes (continue) ☐ No (must reschedule)

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 1 Continued

CONCOMITANT MEDICATIONS LIST

NAME	Dose	Start date	End date	Reason

ANTHROPOMETRICS AND VITAL SIGNS

Pulse rate (bpm) _____ Blood pressure: _____ / _____ (mmHg)
(Seated 5m rest) systolic / diastolic

Height: ____.___ X 2.54 = ____.____ ÷100 = ____.___
in cm m

Weight: ____.___ ÷ 2.2 = ____.___ BMI: _____ (kg/m^2)

FASTING (8-12 hr) BLOOD DRAWN □ Yes □ No

If no, comment: _____

If yes, provide time and date below:

__ __ / __ __ __ / __ __ __ __ ___ ___ : ___ ___ _____
Day Month Year (24 hour clock) Phleb initials

Does subject have any dietary allergies or special dietary needs?
□ Yes □ No

If yes, describe _____

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 1 Continued

SIDE TO BE TESTED (GROUP): RIGHT ____ LEFT ____

ISOMETRIC STRENGTH TEST-KNEE FLEXORS/EXTENSORS:

Leg to be tested: Right _____ Left _____
[Dynamometer orientation=90°, Dynamometer tilt=0°, seat orientation=90°]

Chair Settings:
Front/Back: _____
Height (up/down): _____
Rotation: _____

Seat settings:
Back Fore/Aft: _____
Tilt: _____

Dynamometer Settings:
Left/Right: _____
Height: _____

Attachment length: _____

Subject performed 1-2 SUBMAXIMAL contractions (about 10% effort) at all speeds to familiarize with procedures ☐ Yes ☐ No

Have any adverse events occurred during *this* study visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

PROGRESS NOTES:

_____

_____

_____

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 1 Continued

NEXT STUDY APPOINTMENT

Date of Next Visit: __ __ / __ __ __ / __ __ __ __ Time of Visit: __ __ :__ __
Day Month Year (24 hour clock)

Note: Must be ≥2 days and ≤4 days after Visit 1 and between 11:00-15:00

Was subject instructed to not consume any non-steroidal anti-inflammatory medicine or aspirin containing medicine, alcohol, or caffeine? ☐ Yes ☐ No

Visit Data Collected By:

_____	_____	_____
Printed Name	Signature	Date
_____	_____	_____
Printed Name	Signature	Date

END OF VISIT

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 2

Time of visit: __ __:__ __
(24-hour clock)

Does subject continue to meet all Inclusion/Exclusion criteria? □ Yes □ No
If no, describe _____

Has subject had a cold in the past 24 hours?
□ Yes (must reschedule) □ No (continue)

Has subject consumed any non-steroidal anti-inflammatory medicine or aspirin containing medicine in the past 24 hours?
□ Yes (must reschedule) □ No (continue)

Has subject consumed any alcohol in the past 24 hours?
□ Yes (must reschedule) □ No (continue)

Has subject consumed any caffeine in the past 24 hours?
□ Yes (must reschedule) □ No (continue)

CIRCLE LEG TO BE TESTED AT VISIT 2: RIGHT / LEFT

***Verify it is the same leg as in Visit 1 (See CRF pg. 6)**

ISOMETRIC STRENGTH TEST-KNEE FLEXORS/EXTENSORS:

Leg to be tested: Right _____ Left _____
[Dynamometer orientation=90°, Dynamometer tilt=0°, seat orientation=90°]

Chair Settings:
Front/Back: _____
Height (up/down): _____
Rotation: _____

Seat settings:
Back Fore/Aft: _____
Tilt: _____

Dynamometer Settings:
Left/Right: _____
Height: _____

Attachment length: _____

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __

Day Month Year

VISIT 2 Continued

# OF REPS: 3		EXTENSION AWAY 70°	FLEXION TOWARD 70°
PEAK TORQUE	N-M		
AVG PEAK TORQUE	N-M		
COEFF. OF VAR.	%		

*Trainer Comments: _____

□ **Subject rested for 5 minutes**

ISOKINETIC STRENGTH TEST-KNEE FLEXORS/EXTENSORS:

****Chair Settings will remain the same for all knee flexor/extensor muscle testing.**

# OF REPS: 3		EXTENSION 60°/SEC	FLEXION 60°/SEC
PEAK TORQUE	N-M		
MAX REP TOT WORK	J		
COEFF. OF VAR.	%		
AVG POWER	WATTS		
AVG PEAK TORQUE	N-M		

□ **Subject rested for 2 minutes**

# OF REPS: 5		EXTENSION 180°/SEC	FLEXION 180°/SEC
PEAK TORQUE	N-M		
MAX REP TOT WORK	J		
COEFF. OF VAR.	%		
AVG POWER	WATTS		
AVG PEAK TORQUE	N-M		

*Trainer Comments: _____

□ **Subject rested for 5 minutes**

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 2 Continued

CIRCLE LEG TO BE EXERCISED: RIGHT / LEFT

EXERCISE USING ECCENTRIC CONTRACTIONS:

100 maximal isokinetic eccentric contractions at 90°/sec using the Biodex dynamometer: 10 sets of 10 reps with 10s rest between reps and 1 min rest between sets.
***Check the box below once the set is finished:**

Set 1□ Set 2□ Set 3□ Set 4□ Set 5□

Set 6□ Set 7□ Set 8□ Set 9□ Set 10□

Range of Motion (ROM):_____ Time of completion of exercise session: __ __:__ __
(24-hour clock)

*Trainer Comments: _____

□ **Subject rested for 5 minutes**

POST-EXERCISE MEASURES: ISOMETRIC STRENGTH TEST-KNEE FLEXORS/EXTENSORS

# OF REPS: 3		EXTENSION AWAY 70°	FLEXION TOWARD 70°
PEAK TORQUE	N-M		
AVG PEAK TORQUE	N-M		
COEFF. OF VAR.	%		

*Trainer Comments: _____

□ **Subject rested for 5 minutes**

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 2 Continued

ISOKINETIC STRENGTH TEST-KNEE FLEXORS/EXTENSORS:

****Chair Settings will remain the same for all knee flexor/extensor muscle testing.**

# OF REPS: 3		EXTENSION 60°/SEC	FLEXION 60°/SEC
PEAK TORQUE	N-M		
MAX REP TOT WORK	J		
COEFF. OF VAR.	%		
AVG POWER	WATTS		
AVG PEAK TORQUE	N-M		

*Trainer Comments: _____

☐ **Subject rested for 2 minutes**

# OF REPS: 5		EXTENSION 180°/SEC	FLEXION 180°/SEC
PEAK TORQUE	N-M		
MAX REP TOT WORK	J		
COEFF. OF VAR.	%		
AVG POWER	WATTS		
AVG PEAK TORQUE	N-M		

****Biodex System4 program "Comprehensive Evaluation" reports printed and filled with source documents for current visit:**

Pre-exercise Knee Isometric report: ☐ Yes ☐ No

Pre-exercise Knee Isokinetic Report: ☐ Yes ☐ No

Exercise Report: ☐ Yes ☐ No

Post-exercise Knee Isometric report: ☐ Yes ☐ No

Post-exercise Knee Isokinetic Report: ☐ Yes ☐ No

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 2 Continued

Have any adverse events occurred during *this* study visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

PROGRESS NOTES:

_____

_____

_____

_____

NEXT STUDY APPOINTMENT

Date of Next Visit: __ __ / __ __ __ / __ __ __ __ Time of Visit: __ __ :__ __
Day Month Year (24 hour clock)

Note: Must begin 20 hours after Visit 2 and between 8:00-12:00

Was subject instructed to not consume any non-steroidal anti-inflammatory medicine or aspirin containing medicine, alcohol, or caffeine?
☐ Yes ☐ No

Was subject instructed to not eat breakfast (fast at least 8 hours) before coming to the lab for visit 3? ☐ Yes ☐ No

Visit Data Collected By:

_____ _____ _____
Printed Name Signature Date

_____ _____ _____
Printed Name Signature Date

END OF VISIT

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 3

Time of visit: __ __:__ __
(24-hour clock)

Does subject continue to meet all Inclusion/Exclusion criteria? ☐ Yes ☐ No

If no, describe _____

Has subject had a cold in the past 24 hours?
☐ Yes (subject DQ'd) ☐ No (continue)

Has subject consumed any non-steroidal anti-inflammatory medicine or aspirin containing medicine in the past 24 hours?
☐ Yes (subject DQ'd) ☐ No (continue)

Has subject consumed any calories in the past 8 hours?
☐ Yes (subject DQ'd) ☐ No (continue)

Has subject consumed any alcohol in the past 24 hours?
☐ Yes (subject DQ'd) ☐ No (continue)

Has subject consumed any caffeine in the past 24 hours?
☐ Yes (subject DQ'd) ☐ No (continue)

FASTING (8-12 hr) BLOOD DRAWN ☐ Yes ☐ No
If no, comment: _____

If yes, provide time and date below:

__ __ / __ __ __ / __ __ __ __ ___ ___ : ___ ___ _____
Day Month Year (24 hour clock) Phleb initials

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 3 Continued

ISOMETRIC STRENGTH TEST-KNEE FLEXORS/EXTENSORS:

Leg to be tested: Right _____ Left _____
[Dynamometer orientation=90°, Dynamometer tilt=0°, seat orientation=90°]

Chair Settings:
Front/Back: _____
Height (up/down): _____
Rotation: _____

Seat settings:
Back Fore/Aft: _____
Tilt: _____

Dynamometer Settings:
Left/Right: _____
Height: _____

Attachment length: _____

# OF REPS: 3		EXTENSION AWAY 70°	FLEXION TOWARD 70°
PEAK TORQUE	N-M		
AVG PEAK TORQUE	N-M		
COEFF. OF VAR.	%		

*Trainer Comments: _____

☐ **Subject rested for 5 minutes**

ISOKINETIC STRENGTH TEST-KNEE FLEXORS/EXTENSORS:

****Chair Settings will remain the same for all knee flexor/extensor muscle testing.**

# OF REPS: 3		EXTENSION 60°/SEC	FLEXION 60°/SEC
PEAK TORQUE	N-M		
MAX REP TOT WORK	J		
COEFF. OF VAR.	%		
AVG POWER	WATTS		
AVG PEAK TORQUE	N-M		

☐ **Subject rested for 2 minutes**

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 3 Continued

# OF REPS: 5		EXTENSION 180°/SEC	FLEXION 180°/SEC
PEAK TORQUE	**N-M**		
MAX REP TOT WORK	**J**		
COEFF. OF VAR.	**%**		
AVG POWER	**WATTS**		
AVG PEAK TORQUE	**N-M**		

*Trainer Comments: _____

☐ Subject rested for 5 minutes

****Biodex System4 program "Comprehensive Evaluation" reports printed and filled with source documents for current visit:**

Pre-exercise Knee Isometric report: ☐ Yes ☐ No

Pre-exercise Knee Isokinetic Report: ☐ Yes ☐ No

Have any adverse events occurred during *this* study visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

PROGRESS NOTES:

_____

_____

_____

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 3 Continued

NEXT STUDY APPOINTMENT

Date of Next Visit: __ __ / __ __ __ / __ __ __ __ Time of Visit: __ __ :__ __
Day Month Year (24 hour clock)

Note: Must begin 45-46 hours after Visit 2 (24 hours after Visit 3) and between 8:00-12:00

Was subject instructed to not consume any non-steroidal anti-inflammatory medicine or aspirin containing medicine, alcohol, or caffeine?

☐ Yes ☐ No

Was subject instructed to not eat breakfast (fast at least 8 hours) before coming to the lab for visit 4?

☐ Yes ☐ No

Visit Data Collected By:

_____	_____	_____
Printed Name	Signature	Date
_____	_____	_____
Printed Name	Signature	Date

END OF VISIT

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 4

Time of visit: __ __:__ __
(24-hour clock)

Does subject continue to meet all Inclusion/Exclusion criteria? ☐ Yes ☐ No
If no, describe _____

Has subject had a cold in the past 24 hours?
☐ Yes (subject DQ'd) ☐ No (continue)

Has subject consumed any non-steroidal anti-inflammatory medicine or aspirin containing medicine in the past 48 hours?
☐ Yes (subject DQ'd) ☐ No (continue)

Has subject consumed any calories in the past 8 hours?
☐ Yes (subject DQ'd) ☐ No (continue)

Has subject consumed any alcohol in the past 24 hours?
☐ Yes (subject DQ'd) ☐ No (continue)

Has subject consumed any caffeine in the past 24 hours?
☐ Yes (subject DQ'd) ☐ No (continue)

Standardized breakfast given in lab, If "YES", Time eaten **: __ __:__ __**
(24-hour clock)

Staff initials_____ **Date: __ __ / __ __ __ / __ __ __ __**
DD MMM YYYY

Subject rested in laboratory for 3-4 hours? ☐ Yes ☐ No

Muscle biopsy taken (3-4 hours after meal)? ☐ Yes ☐ No

If "YES", Time of Biopsy: __ __:__ __
(24-hour clock)

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 4 Continued

*Physician Comments: _____

_____

_____

_____

_____

Physician Signature // Date

Have any adverse events occurred during *this* study visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

PROGRESS NOTES:

_____

_____

NEXT STUDY APPOINTMENT

Date of Next Visit: __ __ / __ __ __ / __ __ __ __ Time of Visit: __ __ :__ __
Day Month Year (24 hour clock)

Note: Must be 48 hours after biopsy and between 12:00-16:00

Was subject instructed to not consume any non-steroidal anti-inflammatory medicine or aspirin containing medicine (unless instructed by staff or physician), alcohol, or caffeine? ☐ Yes ☐ No

Care of biopsy sheet explained and given to subject? ☐ Yes ☐ No

Visit Data Collected By:

_____ _____ _____
Printed Name Signature Date

_____ _____ _____
Printed Name Signature Date

END OF VISIT

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

PHONE FOLLOW UP 1: EVENING POST-BIOPSY

Time of contact: __ __:__ __
(24-hour clock)

HAS ANYTHING CHANGED SINCE VISIT 4? ☐ Yes ☐ No
If yes, what has changed? _____

Have any adverse events occurred since the last visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

Date and time of next follow up: __ __ / __ __ __ / __ __ __ __ __ __:__ __
Day Month Year (24-hour clock)

PROGRESS NOTES:

_____
_____
_____

END OF PHONE FOLLOW UP

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

PHONE FOLLOW UP 2: 1 DAY POST-BIOPSY

Time of contact: __ __:__ __
(24-hour clock)

HAS ANYTHING CHANGED SINCE VISIT 4? ☐ Yes ☐ No
If yes, what has changed? _____

Have any adverse events occurred since last contacted? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

Verified scheduled visit 5? ☐ Yes ☐ No

Date and time of visit 5: __ __ / __ __ __ / __ __ __ __ __ __:__ __
Day Month Year (24-hour clock)

***Subject reminded not to consume any caffeine, alcohol, or medications that have anti-inflammatory properties (unless recommended by staff or physician—may include cold/flu medications, NSAIDS)?

☐ Yes ☐ No

PROGRESS NOTES:

_____

_____

_____

END OF PHONE FOLLOW UP

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 5

Time of visit: __ __:__ __
(24-hour clock)

Does subject continue to meet all Inclusion/Exclusion criteria? ☐ Yes ☐ No
If no, describe _____

Has subject had a cold in the past 24 hours?
☐ Yes ☐ No (continue)
If yes, when did symptoms begin?_____
Can subject continue with study? ☐ Yes ☐ No

Has subject consumed any non-steroidal anti-inflammatory medicine or aspirin containing medicine in the past 24 hours?
☐ Yes ☐ No (continue)
If yes, quantity and time(s) _____

Has subject consumed any alcohol in the past 24 hours?
☐ Yes ☐ No (continue)
If yes, quantity and time(s) _____

Has subject consumed any caffeine in the past 24 hours?
☐ Yes ☐ No (continue)
If yes, quantity and time(s) _____

Quick biopsy sites check: ☐ Yes ☐ No
Sites look OK: ☐ Yes ☐ No
Comments_____
_____
_____

Biopsy check initials_____

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 5 Continued

Group: RIGHT ____ LEFT ____

ISOMETRIC STRENGTH TEST-KNEE FLEXORS/EXTENSORS:

Leg to be tested: Right _____ Left _____
[Dynamometer orientation=90°, Dynamometer tilt=0°, seat orientation=90°]

Chair Settings:
Front/Back: _____
Height (up/down): _____
Rotation: _____

Seat settings:
Back Fore/Aft: _____
Tilt: _____

Dynamometer Settings:

Left/Right: _____
Height: _____

Attachment length: _____

# OF REPS: 3		EXTENSION AWAY 70°	FLEXION TOWARD 70°
PEAK TORQUE	N-M		
AVG PEAK TORQUE	N-M		
COEFF. OF VAR.	%		

*Trainer Comments: _____

☐ **Subject rested for 5 minutes**

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 5 Continued

ISOKINETIC STRENGTH TEST-KNEE FLEXORS/EXTENSORS:

****Chair Settings will remain the same for all knee flexor/extensor muscle testing.**

# OF REPS: 3		EXTENSION 60°/SEC	FLEXION 60°/SEC
PEAK TORQUE	N-M		
MAX REP TOT WORK	J		
COEFF. OF VAR.	%		
AVG POWER	WATTS		
AVG PEAK TORQUE	N-M		

☐ **Subject rested for 2 minutes**

# OF REPS: 5		EXTENSION 180°/SEC	FLEXION 180°/SEC
PEAK TORQUE	N-M		
MAX REP TOT WORK	J		
COEFF. OF VAR.	%		
AVG POWER	WATTS		
AVG PEAK TORQUE	N-M		

*Trainer Comments: _____

****Biodex System4 program "Comprehensive Evaluation" reports printed and filled with source documents for current visit:**

Knee Isometric Report: ☐ Yes ☐ No

Knee Isokinetic Report: ☐ Yes ☐ No

Have any adverse events occurred during *this* study visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 5 Continued

PROGRESS NOTES:

_____

_____

_____

NEXT STUDY APPOINTMENT

***Subject reminded not to consume any caffeine, alcohol, or medications that have anti-inflammatory properties (unless recommended by staff or physician—may include cold/flu medications, NSAIDS)?

☐ Yes ☐ No

Date and time of visit 6: __ __ / __ __ __ / __ __ __ __ __ __:__ __
Day Month Year (24-hour clock)

Note: Must be 1 week after Visit 4 and between 12:00-16:00

Date and time of phone follow up (1 day): __ __ / __ __ __ / __ __ __ __ __ __:__ __
Day Month Year (24-hour clock)

Visit Data Collected By:

_____ _____ _____
Printed Name Signature Date

_____ _____ _____
Printed Name Signature Date

END OF VISIT

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

PHONE FOLLOW UP 3: 3 DAYS POST-BIOPSY

Time of contact: __ __:__ __
(24-hour clock)

HAS ANYTHING CHANGED SINCE VISIT 5? ☐ Yes ☐ No
If yes, what has changed? _____

Have any adverse events occurred since the last visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

***Subject reminded not to consume any caffeine, alcohol, or medications that have anti-inflammatory properties unless recommended by staff or physician (may include cold/flu medications, NSAIDS)?

☐ Yes ☐ No

Date and time of next follow up (1 day): __ __ / __ __ __ / __ __ __ __ __ __:__ __
Day Month Year (24-hour clock)

PROGRESS NOTES:

_____
_____
_____

END OF PHONE FOLLOW UP

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

PHONE FOLLOW UP 4: 4 DAYS POST-BIOPSY

Time of contact: __ __:__ __
(24-hour clock)

HAS ANYTHING CHANGED SINCE VISIT 5? ☐ Yes ☐ No
If yes, what has changed? _____

Have any adverse events occurred since the last visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

***Subject reminded not to consume any caffeine, alcohol, or medications that have anti-inflammatory properties (unless recommended by staff or physician—may include cold/flu medications, NSAIDS)?

☐ Yes ☐ No

Date and time of next follow up (1 day): __ __ / __ __ __ / __ __ __ __ __ __:__ __
Day Month Year (24-hour clock)

PROGRESS NOTES:

_____

_____

_____

END OF PHONE FOLLOW UP

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

PHONE FOLLOW UP 5: 5 DAYS POST-BIOPSY

Time of contact: __ __:__ __
(24-hour clock)

HAS ANYTHING CHANGED SINCE VISIT 5? ☐ Yes ☐ No
If yes, what has changed? _____

Have any adverse events occurred since the last visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

***Subject reminded not to consume any caffeine, alcohol, or medications that have anti-inflammatory properties (unless recommended by staff or physician—may include cold/flu medications, NSAIDS)?

☐ Yes ☐ No

Date and time of next follow up (1 day): __ __ / __ __ __ / __ __ __ __ __ __:__ __
Day Month Year (24-hour clock)

PROGRESS NOTES:

_____

_____

_____

END OF PHONE FOLLOW UP

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

PHONE FOLLOW UP 6: 6 DAYS POST-BIOPSY

Time of contact: __ __:__ __
(24-hour clock)

HAS ANYTHING CHANGED SINCE VISIT 5? ☐ Yes ☐ No
If yes, what has changed? _____

Have any adverse events occurred since the last visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

***Subject reminded not to consume any caffeine, alcohol, or medications that have anti-inflammatory properties (unless recommended by staff or physician—may include cold/flu medications, NSAIDS)?

☐ Yes ☐ No

Verified scheduled visit 6? ☐ Yes ☐ No

Date and time of visit 6: __ __ / __ __ __ / __ __ __ __ __ __:__ __
Day Month Year (24-hour clock)

PROGRESS NOTES:

_____
_____
_____

END OF PHONE FOLLOW UP

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 6

Time of visit: __ __:__ __
(24-hour clock)

Does subject continue to meet all Inclusion/Exclusion criteria? ☐ Yes ☐ No
If no, describe _____

Has subject had a cold in the past 24 hours?
☐ Yes ☐ No (continue)
If yes, when did symptoms begin?_____
Can subject continue with study? ☐ Yes ☐ No

Has subject consumed any non-steroidal anti-inflammatory medicine or aspirin containing medicine in the past 24 hours?
☐ Yes ☐ No (continue)
If yes, quantity and time(s) _____

Has subject consumed any alcohol in the past 24 hours?
☐ Yes ☐ No (continue)
If yes, quantity and time(s) _____

Has subject consumed any caffeine in the past 24 hours?
☐ Yes ☐ No (continue)
If yes, quantity and time(s) _____

Quick biopsy sites check: ☐ Yes ☐ No
Sites look OK: ☐ Yes ☐ No
Comments_____
_____
_____

Biopsy check initials_____

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 6 Continued

Group: RIGHT ____ LEFT ____

ISOMETRIC STRENGTH TEST-KNEE FLEXORS/EXTENSORS:

Leg to be tested: Right _____ Left _____
[Dynamometer orientation=90°, Dynamometer tilt=0°, seat orientation=90°]

Chair Settings:
Front/Back: _____
Height (up/down): _____
Rotation: _____

Seat settings:
Back Fore/Aft: _____
Tilt: _____

Dynamometer Settings:

Left/Right: _____
Height: _____

Attachment length: _____

# OF REPS: 3		EXTENSION AWAY 70°	FLEXION TOWARD 70°
PEAK TORQUE	N-M		
AVG PEAK TORQUE	N-M		
COEFF. OF VAR.	%		

*Trainer Comments: _____

☐ **Subject rested for 5 minutes**

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 6 Continued

ISOKINETIC STRENGTH TEST-KNEE FLEXORS/EXTENSORS:

****Chair Settings will remain the same for all knee flexor/extensor muscle testing.**

# OF REPS: 3		EXTENSION 60°/SEC	FLEXION 60°/SEC
PEAK TORQUE	N-M		
MAX REP TOT WORK	J		
COEFF. OF VAR.	%		
AVG POWER	WATTS		
AVG PEAK TORQUE	N-M		

☐ **Subject rested for 2 minutes**

# OF REPS: 5		EXTENSION 180°/SEC	FLEXION 180°/SEC
PEAK TORQUE	N-M		
MAX REP TOT WORK	J		
COEFF. OF VAR.	%		
AVG POWER	WATTS		
AVG PEAK TORQUE	N-M		

*Trainer Comments: _____

****Biodex System4 program "Comprehensive Evaluation" reports printed and filled with source documents for current visit:**

Knee Isometric Report: ☐ Yes ☐ No

Knee Isokinetic Report: ☐ Yes ☐ No

Have any adverse events occurred during *this* study visit? ☐ Yes ☐ No

If yes, please comment in progress notes and record on the appropriate adverse event page.

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

VISIT 6 Continued

PROGRESS NOTES:

_____

_____

_____

Sutures removed? ☐ Yes ☐ No

If "YES", Time: __ __:__ __
(24-hour clock)

Physician Signature // Date

*Physician Comments: _____

NEXT STUDY APPOINTMENT—ONLY IF SUTURES NOT REMOVED AT THIS VISIT

Date of Next Visit: __ __ / __ __ __ / __ __ __ __ Time of Visit: __ __ :__ __
Day Month Year (24 hour clock)

Visit Data Collected By:

_____	_____	_____
Printed Name	Signature	Date

_____	_____	_____
Printed Name	Signature	Date

END OF VISIT

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

SUTURE REMOVAL

Date of Visit: __ __ / __ __ __ / __ __ __ __
Day Month Year

Sutures removed? ☐ Yes ☐ No

If "YES", Time: __ __:__ __
(24-hour clock)

Physician Signature // Date

*Physician Comments: _____

Visit Data Collected By:

_____	_____	_____
Printed Name	Signature	Date

_____	_____	_____
Printed Name	Signature	Date

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

Procedure Evaluation

Please rate the difficulty in completing the tasks below by checking the appropriate box. NOTE: 1 = simple, 5= very difficult

	Simple 1	2	3	4	Very Difficult 5
Eccentric Exercise					
Isometric strength tests					
Isokinetic strength tests					
Ability to rate soreness					

END OF STUDY

Did subject complete study per protocol? □ **Yes** □ **No**

If "no," provide reason:

Screen Failure ____
Protocol violation ____
Adverse event ____
Withdrew consent ____
Other ____ **specify:**

Comments:_____
_____
_____
_____
_____

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

CONCOMITANT MEDICATIONS

List all medications (OTC and supplements included) the subject took in the past 35 days and/or during the study.

Check here if none ____

NAME	Dose/ route	Start date	End date	Cont

SUBJECT NUMBER ___ ___

GROUP (R/L) _____ DATE OF VISIT __ __ / __ __ __ / __ __ __ __
Day Month Year

ADVERSE EVENTS

List all adverse events the subject experiences since signing the informed consent document

Check here if none _____

Event	Intensity: mild, moderate, or severe	Start date	End date	Cont	Study related: no, possibly, or yes	Treatment	Serious Yes/No

APPENDIX B: Standard Operating Procedures

SMOKING STUDY BLOOD COLLECTION

Blood is collected at V1 and V3, same procedure each time, same time of day.

For Phlebotomist:

- Blood can be drawn from either arm.
- Phlebotomist draws the following tubes in the following order:
    - TWO RED TUBES
    - TWO PURPLE (EDTA) TUBES
    - ONE GREEN (NaHep) TUBE

- Invert the purple and green tubes several times and leave on aliquoting station. If no assistant immediately available, please write the time of draw on the tubes and start a timer for 15 minutes.

For Assistant:

- Set up labeler before draw or while centrifugation. Label as follows:
- Tube type key: SERUM = red top; EDTA = purple; NAHEP = green

SMOKE-(subject number)—(initials)
(DATE)—(TIME)
(V#)—(Tube type)

Example:
SMOKE-13—NAM
1/01/01—8:00AM
V1—SERUM

- Aliquot containers are snap caps—500uL per cap. Take as many samples as possible.
- Once drawn, RED tops need to sit for 30 minutes, then centrifuged at 1000Xg for 15 min.
- PURPLE and GREEN invert several times, let sit for 15 minutes, then centrifuge 1000Xg for 10min.

Muscular Strength Test and Eccentric Exercise

SUMMARY:
Testing procedures will occur as follows:

- **Warm-up (walking, 5 minutes)**
- **Pre-exercise strength testing**
  - **Isometric strength test**
    - **5 minutes rest**
  - **Isokinetic strength test, 60°/sec**
    - **2 minutes rest**
  - **Isokinetic strength test, 180°/sec**
    - **5 minutes rest**
- **Eccentric exercise**
  - **5 minutes rest**
- **Post-exercise strength testing**
  - **Isometric strength**
    - **5 minutes rest**
  - **Isokinetic strength, 60°/sec**
    - **2 minutes rest**
  - **Isokinetic strength, 180°/sec**

Lower body muscular strength tests and eccentric exercise taken by:

- ❑ Knee extension isometric force at 70°
- ❑ Knee isokinetic force (flexion/extension) at 60°/sec and 180°/sec
- ❑ Muscle exercise consists of 100 maximal isokinetic eccentric contractions at 90°/sec using the Biodex dynamometer. There are 10 sets of 10 reps with 10s rest between reps and 1 min rest between sets.
- ❑ All force variables will be reported in metric units:
  - **Peak Torque** measured in Newton-meters (Nm) = highest muscular force output at any moment during a repetition; indicative of strength capacity.
  - **Work** measured in Joules (J) = indicative of capability to produce force throughout the ROM. "**MAX REP TOT WORK**" is the total muscular force output for the repetition with the greatest amount of work.
  - **Average Power** measured in watts = total work divided by time; represents how quickly a muscle can produce force.

**** <u>Before the testing procedures, subjects will complete a warm-up period consisting of submaximal walking on a treadmill (2.5-3.0 mph) for 5 minutes.</u>**

General Biodex Program Set-Up For All Muscle Strength Tests & Exercises

- ❑ Switch Biodex System 4 Isokinetic dynamometer on by turning on master switch and 2 auxiliary switches located at the rear of the computer console.

- ❑ Turn on computer tower and monitor (located on side of console).

- ❑ Make sure all attachments are removed from dynamometer and click **OK** to initialize dynamometer.

- ❑ Open **Biodex Advantage** Program.

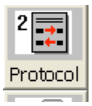
- ❑ **TO ADD A NEW PATIENT:**

1. Click on "**Patient**" tab and add a new patient.
2. Fill out all demographic fields.
3. Check "**None**" under "**Involved**."
4. When finished filling out all fields, click "**Save**" on top toolbar to save patient demographic information.

- ❑ **TO OPEN AN EXISTING PATIENT FILE** (FOR PREVIOUSLY TESTED SUBJECTS)**:**

1. Click on "**Patient**" tab choose from the list of patients.
2. Select a previous test or choose "**NEW**" to initialize a new test (for testing after Visit 1).

- ❑ Click "**Protocol**" button on TOP toolbar



*****if adding a NEW PATIENT you must first click the "protocol" button on the SIDE toolbar and then the "protocol" button on the top tab and select the appropriate test.

Standardized Protocols have been generated for all tests and exercises and are labeled as follows:

- **Smoking Isometric**
- **Smoking Isokinetic**
- **Smoking exercise**

Protocols are located as sub-headings under their respective major headings of "**ISOMETRIC UNILATERAL**" and "**ISOKINETIC UNILATERAL.**"

- ❑ **SUBJECT POSITION:**
- ❑ Constant settings for ALL subjects:
  Dynamometer height = 0, Dynamometer orientation = 90°,

Dynamometer tilt = 0°, Seat orientation = 90°

- ❑ Subjects will be seated with the torso at 90 degrees of hip flexion and knees flexed at approximately 90 degrees.

1) Rotate seat using the lever on the underside of the seat such that the leg to be tested is on the same side as the dynamometer.

2) Push the dynamometer to the far end on the side of the leg being tested (~17-18 on the scale). The dynamometer can move right/left by first stepping on the corresponding pedals (gray) and then pushing or pulling the dynamometer into place.

3) Attach knee flexion attachment and secure it to dynamometer by tightening the knob. Make sure to line up dots.

4) Adjust dynamometer height. Dynamometer height = 0. Adjust the height of the dynamometer by rotating the lever (located midway up the dynamometer stand), and lifting or lowering it (it is on a spring).

5) Have subject sit on chair and adjust **Seat Position (front/back)**. To do this, rotate crank (located behind the seat back) so that the seatback rests firmly against the lower back while the edge of the seat maintains contact with the subject's upper calf muscle.

6) Adjust **Chair Front/Back** and **Chair Height**. The height of the seat is motorized and can be adjusted up or down by stepping on one of the black circular pedals located at the base of the seat. The chair can be moved front/back by stepping on its corresponding pedals (gray) and then pushing or pulling.

7) Place subject's leg in the knee flexion attachment without securing the straps and adjust height and position of the chair such that the ***lateral femoral epicondyle is aligned with the axis of rotation of the lever arm.***

8) After positioning the chair, position the **Attachment Length**. Bring the knee angle to about 45 degrees and lock the dynamometer. With the subject's leg resting on the pad of the knee flexion attachment, instruct them to plantar flex the ankle ("point your toe"). Adjust the length so that the bottom of the pad is just above and not touching the heel. Secure the top pad around the ankle; making sure that it is very snug, but not uncomfortably tight for the subject.

9) Make any necessary changes to the **Dynamometer's Right/Left** position.

10) Secure the subject with both Velcro straps criss-crossed over the chest/torso, at the pelvis, and thigh to prevent extraneous movement. Both arms should be folded across the chest during the test trials.

11) Record all positioning reference numbers in the CRF for future tests.

12) Return to general set-up to set ROM.

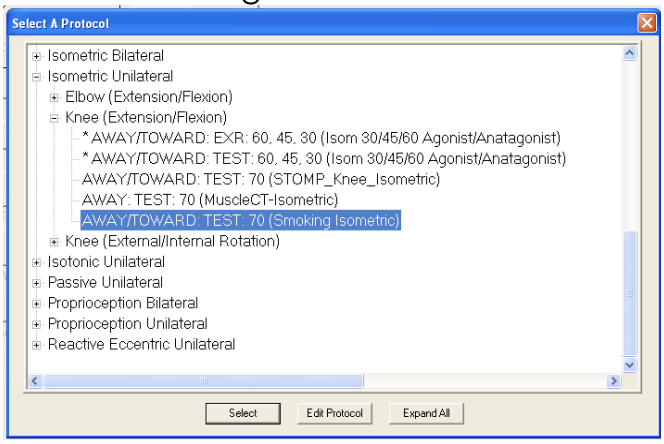
- ❑ Select a previous test or choose "**NEW**" to initialize a new test

Knee Strength Assessment

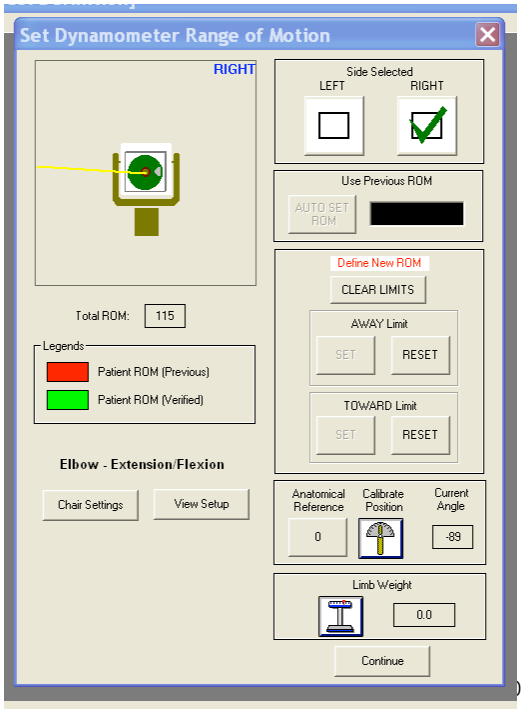
***** During Visit 1 subjects will be positioned in the Biodex and perform 1 very light contraction to familiarize them with the tasks. At Visits 2, 5, and 6 subjects will perform the full isometric/isokinetic strength testing. During Visit 2, isometric/isokinetic strength testing will be performed before and after eccentric exercise.***

Isometric Test

- ❑ Open the "**Smoking Isometric**" protocol:
  1. Click the "**Patient**" button in the upper left hand side of the screen.
  2. Select "**Open**" and find the subject's name. Click on the subjects name and select "**NEW**" at the bottom left of the screen. To select the protocol, click "**Protocol**" on the top toolbar and find "**Smoking Isometric**" under the major subheadings of "**ISOMETRIC UNILATERAL**" and "**KNEE (EXTENSION/FLEXION)**"



- ❑ Open the "**Smoking Isometric**" protocol , Click "**Select**"
  In "**Set Dynamometer Range of Motion**" window:



- ❑ Select leg to be tested.

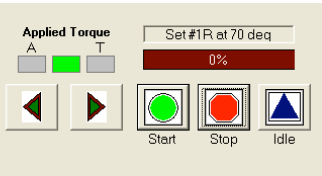
- ❑ Select "**CLEAR LIMITS**".

- ❑ Set ROM limits:
  1. Bring subject's leg into full extension and push the black **"HOLD/RESUME"** button located on the dynamometer control panel (between the two blue and red buttons). This will lock the knee in the fully extended position.
  2. Click "**SET**" under "**AWAY LIMIT**."
  3. Set the toward limit by moving the limb 95 degrees from the extended position. Use the "Range of Motion" box in the upper left hand corner to determine how many degrees the limb has moved. Push the HOLD button to lock the limb in this position and click **"Set"** under **"TOWARD Limit"**.
  4. Move the limb back towards the **"TOWARD Limit"** by 5 degrees and push the HOLD button. The **"Anatomical Reference"** should read 90 degrees. Click "**CALIBRATE POSITION**".
  5. Weigh the limb by placing it in a fully extended position. Press the HOLD button on the dynamometer to lock the limb in this position; ask the subject to relax the limb and click the scale icon *twice* to record limb weight.

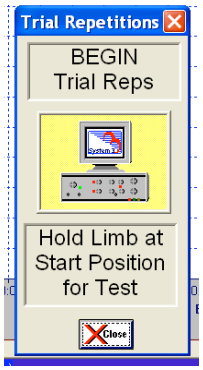
- ❑ Click "**CONTINUE**" to initialize test.

- ❑ Explain to the subject that when you click "Start" the dynamometer will position their leg at a 70 degree angle. When they hear the **"JINGLE"** instruct the subject will warm up by kicking and pulling against the dynamometer arm. Tell the subject about the test—the leg will not move no matter how hard the subject kicks and pulls. There will be a warm up, then kick as hard as possible for 4 seconds, rest for one minute, then pull as hard as possible for 4 seconds. This will be repeated 3 times. Explain that it is crucial that they kick and pull as hard as possible in all tests, as maximal effort is repeatable and submaximal is not.

- ❑ Initialize the test by pushing the "**Start**" button (lower right side of window).



- ❑ This will bring the lever into the starting position of 70 degrees flexion and the "**Trial Repetitions**" window will appear.



- ❑ Instruct subject to begin warm up by kicking in "first gear" or lightly, then pulling in "first gear." Next instruct the subject to kick and pull in "second gear" or moderate intensity—about half of their maximal effort. Finally, instruct the subject to kick and pull in "third gear" or as hard as they can.

- ❑ After the warm up is complete, the Trial Reps Box will remain. Instruct the subject that they will kick away from their body on your command for 4 seconds. Use the cues "kick, kick, kick….." as commands. Also, remind the subject that the lever will not move when they push against it. The subject will do this 3 times with one minute of rest between each repetition.

- ❑ Countdown for the subject from five, clicking the "✖**CLOSE**" button when you say "one" and instructing them to start when you say **"KICK"**.
  For example, **5…4…3…2…1(click "✖CLOSE" button)…KICK!"**.

- ❑ During the one-minute rest period explain that on the next repetition they are to pull their leg as hard as they can towards their body on your command. Use the commands "pull, pull, pull". Give the subject a count down from 5 seconds and tell them to pull when you say **"PULL"**. Repeat this pattern for the next 2 sets.
  For example, **5…4…3…2…1…PULL!"**.

- ❑ At the conclusion of the test select "**Yes**" to end test.

***There will be 5 minutes rest. Remember to write down the chair setting and fill in the resource forms.**

Isokinetic Test

- ❑ Following 5 minutes rest, subjects will perform 3 contractions at 60°/sec (1.05 rad/sec) and 5 contractions at 180°/sec(3.14 rad/sec) through their defined ROM.

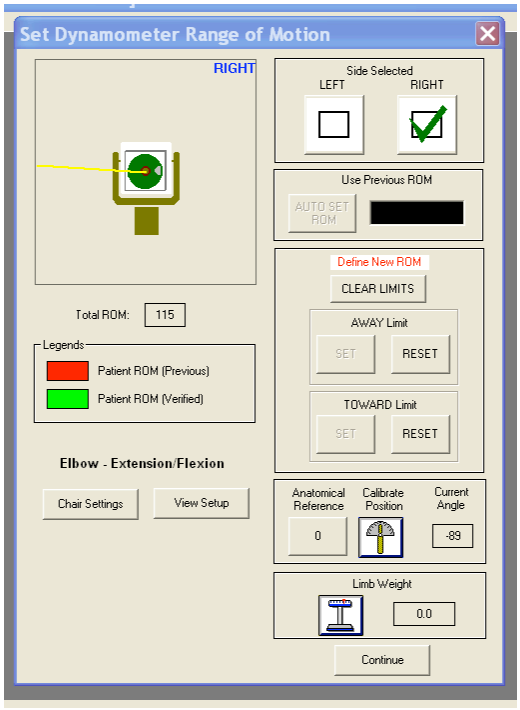
- ❑ 2 minutes rest will be given between sets.

- ❑ Open the "**Smoking Isokinetic**" protocol by clicking the "**Patient**" button in the upper left hand side of the screen.

- ❑ Select "**Open**" and find the subject's name. Click on the subjects name and select "**NEW**" at the bottom left of the screen. To select the protocol, click "**Protocol**" on the top toolbar and find "**Smoking Isokinetic**" under the major subheadings of "**ISOKINETIC UNILATERAL**" and "**KNEE (EXTENSION/FLEXION)**".

- ❑ Open the "**Smoking Isokineticic**" protocol , Click "**Select**"

In "**Set Dynamometer Range of Motion**" window:



- ❑ Select leg to be tested.

- ❑ Select "**CLEAR LIMITS**".

- ❑ Set ROM limits as in isometric test:
  1. Bring subject's leg into full extension and push the black **"HOLD/RESUME"** button located on the dynamometer control panel (between the two blue and red buttons). This will lock the knee in the fully extended position. This position will be the subject's "0" degree angle.
  2. Click "**SET**" under "**AWAY LIMIT**."
  3. Set the toward limit by moving the limb exactly 90 degrees from the extended position. Use the "Range of Motion" box in the upper left hand corner to determine how many degrees the limb has moved. Push the HOLD button to lock the limb in this position and click **"Set"** under **"TOWARD Limit"**.
  4. The **"Anatomical Reference"** should read 90 degrees. Click "**CALIBRATE POSITION**".
  5. Weigh the limb by placing it in a fully extended position. Press the HOLD button on the dynamometer to lock the limb in this position and click the scale icon to record limb weight.

- ❑ Click "**CONTINUE**" to initialize test.

- ❑ Explain to the subject that they will be doing 3 contractions total, starting with the away stroke (extension). Instruct the subject to perform all 3 repetitions in alternating succession (kick, pull, kick, pull, etc.) at 100% of their maximum effort. Click the "**Start**" button and the subject will perform 3 warm-up reps – "1st gear" (minimal effort), "2nd gear" (medium effort), and "3rd gear" (maximum effort). When the warm-up reps are complete, instruct the subject to bring the knee to the 90 degree position and begin when they hear the **HORN blow**. As a tester, after the warm-up reps you can hold the leg still until the horn sounds. Data will not be collected until the subject starts to move.

- ❑ The rest period will be 2 minutes between sets. During this period instruct the subject that during the next set, the contractions will be done faster.

- ❑ At the conclusion of the rest period, the Trial Reps Box will appear and the jingle will sound. Instruct the subject to perform 3 more warm-up reps (1st gear, 2nd gear, and 3rd gear). This will allow the subject to get acquainted with the faster

speed. When they complete the warm-up reps, instruct the subject to bring the knee to the 90 degree position and begin when they hear the horn. As a tester, after the warm-up reps you can hold the leg still until the horn sounds. Data will not be collected until the subject starts to move.

Knee Extensor Muscle Eccentric Exercise

*****At Visit 2, subjects will perform the exercise after* pre-exercise *isometric/isokinetic strength testing.***

*****There will be 5 minutes rest after the pre-exercise isokinetic test.***

- ❑ Open the "**Smoking Exercise**" protocol by clicking the "**Patient**" button in the upper left hand side of the screen.

- ❑ Select "**Open**" and find the subject's name. Click on the subjects name and select "**NEW**" at the bottom left of the screen. To select the protocol, click "**Protocol**" on the top toolbar and find "**Smoking Exercise**" under the major subheadings of "**ISOKINETIC UNILATERAL**" and "**KNEE (EXTENSION/FLEXION)**".

- ❑ Open the "**Smoking Exercise**" protocol. Click "**Select**".

- ❑ Click **"Set ROM"** on the left screen.

In "**Set Dynamometer Range of Motion**" window:

- ❑ Select leg to be tested.

- ❑ Select "**CLEAR LIMITS**"

Set ROM limits

1. Bring subject's knee into full extension and push the black "HOLD/RESUME" button. This will lock the knee in the fully extended position. This position will be the patient's "0" degree angle.
2. Click on "Anatomical reference calibrate position"(should now be at "0" degrees)
3. Click "HOLD/RESUME" button. Watch "CURRENT ANGLE", move leg to "35" degree. Push the HOLD button to lock the knee in this position and click **"Set"** under **"AWAY LIMIT"**.
4. Click "HOLD/RESUME" button. Instruct the subject to move leg towards the body as furthest as he can. Push the HOLD button to lock the knee in this position and click **"Set"** under "**Toward Limit**."
5. Write down the total ROM in the CRF.

6. Click "**HOLD/RESUME**" button. Move leg back to start position (**35 degree of flexion)**
7. **Set anatomical reference at 35**. Now click "**Calibrate position**".

- ❑ Click "**CONTINUE**" to initialize test.

- ❑ Explain to the subject that they will be doing **100 maximal isokinetic eccentric contractions at 30 deg/sec. There will be 10 sets of 10 reps with 10s rest between reps and 1 min rest between sets.** Instruct the subject to perform each repetition by keeping kicking at 100% of their maximum effort. Explain to the subjects that when you click "Start", they need to position their leg at a 35 degree angle. Tell the subjects to relax the 10s rest between the reps and will say "KICK" when they need to start kicking again.

- ❑ Click the "**Start**". A "**Biodex Advantage**" window will appear. Click "**Yes**".

- ❑ A "**Release Hold**" window will appear. Click "**HOLD/RESUME"** button.

- ❑ As a tester, **you need to hold the leg still until the time you want the subject to start trial repetition**. Countdown for the subject from five, unhold subject's leg and instruct them to start when you say **"KICK"**. Instruct the subject that they will keep kicking in each rep until the machine stops resisting the leg. Instruct and assist the subject by moving the leg back to start position (35 degree position). **Instruct the subject to relax until the time you want the subject to start again.** After the first rep, **you need to instruct the subject to relax the leg still until 10s (tell the time by watching the screen) later when you need to say "KICK".**

*****Pay attention to the subject's position because it will change while they are doing exercise. Watch the subject to be sure that the lateral femoral epicondyle is aligned with the axis of rotation of the lever arm.***

- ❑ There will be 1 min rest between sets.

- ❑ At the conclusion of the test select "**Yes**" to end test.

SUBJECT WILL REST FOR 5 MINUTES, THEN PERFORM ISOMETRIC AND ISOKINETIC TESTING AS BEFORE. BE SURE TO SELECT A NEW TEST FOR THE POST-EXERCISE TESTS AND PRINT ALL REPORTS.

APPENDIX B: Standard Operating Procedures

Muscle Biopsy Procedures Muscle Biology and Imaging Lab

Principal Investigator: Priscilla M. Clarkson

- Muscle biopsies will be taken from the vastus lateralis muscle. The biopsy will be obtained under local anesthesia (Lidocaine) by a licensed physician.
- The procedure is performed in the Muscle Biology & Imaging Laboratory (163 Totman Building) or at the University Health Services.

Surgical Procedure:

1. Assistants will position the subject on the examination table: the subject is asked to lie in a supine (on back) position on a padded examination table. Before the biopsy procedure, the Physician will provide thorough explanation to the subject about procedures and possible risks, etc. (See General Risks and Discomforts Section below).

2. Both of the Assistants and the Physician will wash hands prior to start of procedure and put on gloves.

3. In the case where there is an exercise (or treatment) and a control leg, the exercised leg will be biopsied first.

4. The Physician will select a biopsy site on the medial side of the vastus lateralis muscle belly. During this time, the Assistant will hold the Subject's foot while the subject flexes the quadriceps and dorsiflexes the foot. The biopsy site will be located lateral to the rectus femoris muscle, midway between the hip and knee joints. The Assistants will shave the biopsy area if needed.

5. The Physician will clean the site with antiseptic (e.g. Povidone-Iodine) scrubs and then the skin area is further cleaned by sterilized alcohol pads.

6. The first Assistant will hand the surgical gloves to Physician (inside packaging will serve as a sterile field). After the Physician has donned sterile gloves, using aseptic technique, the Assistant will open items as directed by the Physician (drapes, gauzes, syringes, needles, tubing, forceps, tips, hemostats, etc.).

7. The first Assistant will open the top of the Lidocaine vial and clean with alcohol pad, and hold the vial upside down for physician to withdraw medication. The first Assistant will place the vial on the clean side of counter.

8. After the incision site has dried, The Physician will administer 2% Lidocaine, (total of up to 8-10 cc subcutaneously initially superficial and then deep including muscle). Initially 0.2-0.4cc.of Lidocaine will be injected at several sites immediately adjacent to the intended incision. Each time, the Physician will draw back slightly on syringe to check for venous

puncture. **It is important not to inject Lidocaine into a vein**. During this part of the procedure, the Subject will probably feel a burning sensation as the anesthetic enters the skin and the area under the skin just above the muscle. The Lidocaine may take up to 5 minutes to take effect.

9. When the incision site and surrounding area are numb, The Physician will make an initial incision into the skin and fascia of the muscle. The intention is that the incision will be 1-2 centimeters; however, based on clinical determination at the time of the biopsy, the incision could be 3 cm. During this incision, the Subject should not feel any pain. However they may experience a slight pressure**. Additional Lidocaine** (up to 5cc) may be infused if the Subject reports any pain.

10. After making the initial incision, The Physician will apply pressure using sterile gauze pads to stop bleeding. Once initial bleeding has stopped, the scalpel is reinserted into the skin incision and the Physician will make a larger incision into the fascia of the underlying muscle to be biopsied. **NOTE: During this incision, the Subject may feel slight cramping as the scalpel can stimulate the muscle to contract. After making** the second incision, the Physician will apply pressure using sterile gauze pads to control and stop any bleeding.

11. The first assistant will open the packaging for the biopsy needle, the clear intravenous tubing and a large sterile syringe (e.g. 60-140 ml) used for suction. The Physician will connect the open stopcock end of the tube to the syringe and cut off the other luer-lock end and attach it to the pipette tip that will be inserted into the biopsy needle. **To perform the biopsy:**
    - The Physician will insert the biopsy needle ~5 cm into the incision so that the shaft of the needle is ~perpendicular to the leg. **NOTE: During needle placement and sample collection, the Subject may feel cramping and minor pain or discomfort as the needle can stimulate the muscle to contract**.
    - The Physician will ask the second Assistant to create suction with the attached syringe to draw the muscle into the inner chamber of the needle. The other Assistant may be asked to place pressure on the medial and lateral aspects of the leg to compress the muscle.
    - The Physician will lower the inner blade to cleave the muscle sample.
    - While keeping the needle steady, the Physician will rotate the needle ~120° to reposition for another sample.
    - Once the needle is repositioned, the Physician will raise the blade, have Assistant again create suction and the other Assistant apply pressure as needed. The Physician will lower the blade to collect another sample.
    - This process will be repeated a third time. **NOTE: 3-4 small muscle samples (each the size of a grain of rice) will be collected from each leg.**

12. During sample collection, the needle will remain in the leg in the same vertical position while it is rotated longitudinally through 360° to obtain each sample. However, if a sample is not obtained, the needle may be inserted 2-3 more times. After all samples have been collected, the Physician will withdraw the needle and remove the inner chamber containing

the samples. After the needle is withdrawn, the Physician will apply pressure with sterile gauze to control and stop any bleeding. The first Assistant will determine if the sample size is adequate.

13. When the biopsy is complete, The Physician will hand the biopsy needle & attached tubing containing the muscle samples to the assistant for processing. If needed, the Assistant will flush the end of the inner biopsy needle with sterile saline to transfer additional sample onto a sterile prep for processing (e.g. sectioning and freezing individual samples).

14. While the Assistants are processing the sample, the Physician will maintain pressure on the biopsy site until bleeding stops. When the bleeding stops, the Physician will bring edges of the incision site together and apply sutures as needed to close the incision (if the incision is up to ~1.5 cm, steri-strips may be used instead of sutures).

15. The Physician will apply a pressure bandage: First, sterile gauze pads will be layered directly over the stitches; and an elastic compression bandage is applied around the leg. The subject will be instructed to leave the bandage on overnight. **NOTE: if the bandage becomes too tight or migrates, the subject may loosen or re-position the bandage to a more tolerable level of compression**.

16. The Physician will apply ice packs to the outside of the compression bandage on biopsy sites and cover with cohesive (ACE) bandage. The Subject will be instructed to leave the ice packs and leave in place for at least a couple of hours.

17. The subject will be given a snack (e.g. fruit punch juice box and cheese & cracker snack).

18. Provide the Subject with a "Care of Your Biopsy Incision" instruction sheet (see attached), and a packet of biopsy care supplies including: 2-3 packets of Tylenol, a supply of bandages, and Tegaderm waterproof covering. Instructions in the "Care of Your Biopsy Incision" will be reviewed with the Subject. The subject will be advised that keeping knee as flexed as possible, especially during sleeping, will reduce bleeding and muscle stiffness and aid in recovery after the biopsy. The subject will be advised to use ice for 20 minutes every 2 hours. The subject will also advised against all vigorous activity during the first 48 hours post-biopsy and not to shower or get the incision wet during the first 48 hours post-biopsy. These suggestions should minimize pain and unwarranted bleeding. **NOTE: the area around the biopsy sites will be sore for several days, this is normal and should lessen, however excessive pain or tenderness should be reported to the study coordinators immediately.**

19. The Subject will be driven to their next destination.

General Risks and Discomforts Associated with a Muscle Biopsy:

NOTE: The elements describes below will be discussed with the subject during their interview and restated by the physician immediately prior to the procedure. The subject will be reminded that the procedure will be stopped immediately if they do not wish to continue.

APPENDIX B: Standard Operating Procedures

a) During initial injection of the anesthetic (lidocaine), the subject may experience burning or stinging sensation before the area becomes numb.
b) During the incision and subsequent biopsy, the subject may experience minor pain or discomfort often described as a dull aching or pressure. The scalpel could make the muscle contract or cramp which is normal.
c) After the biopsy is completed the area may be numb for a period of time as the anesthesia wears off. After the anesthetic wears off, the area will likely be sore and tender for a few days. However, this should lessen with time. Excessive pain or discomfort should be reported to study personnel.
d) Potential risk for infection (a slight risk any time the skin is broken)
e) Potential risk for bleeding at the site.
f) Potential risk for bruising of the area, and damage to the muscle tissue or other tissues in the area (rare).
g) Potential risk for hematoma (collection of blood in the tissues outside of a blood vessel) may occur near the skin or in the muscle area. This may cause stiffness and pain in the thigh but this pain should go away within a week. (In very rare conditions this could require surgery).
h) At the biopsy site, a scar will result, but usually this will fade in time. NOTE: **These risks are very low because there are no big blood vessels near the biopsy site and because the muscle tissue usually stops any bleeding by pressing against itself. Also, muscle rapidly repairs itself after the biopsy.** It is possible that the temporary numbness around the biopsy site will last for several days to weeks.

Safety Procedures for Handling Sharps:

No one may touch or manipulate the biopsy tray(s) until the needles and scalpels have been disposed of exclusively by the physician. Staff will verify with the physician that the sharps have been disposed of in the sharps container before disposing of contaminated materials from the tray.

Emergency Procedures in the Event of Exposure to a Contaminated Sharp Object:

In the event of an accidental needle stick or puncture of the skin with a scalpel, the injured party (staff or subject) will immediately notify all staff members and wash area with soap water. Study staff will request the oral consent of the subject (or source of contamination) to be taken to University Health Services to provide a blood sample for HIV/AIDS and Hepatitis B/C. If the injury is life-threatening, study staff will immediately contact 911 and provide directions to the laboratory. If the injury is non-life threatening, a second staff member will immediately assist the injured party and subject (or source of contamination) to University Health Services for medical care, including an HIV/AIDS and Hepatitis B/C screening.

Principal Investigator: Dr. Priscilla Clarkson _____ _____
Signature Date

Study Physician: Dr. Stuart Chipkin _____ _____
Signature Date

APPENDIX B: Standard Operating Procedures

CARE OF YOUR BIOPSY INCISION

Dear Study Participant:

You have had a biopsy of your muscle (s). These instructions are intended to promote healing and speed your recovery. Please review these instructions carefully and ask any questions you may have. Your safety and comfort are very important to us!

ACTIVITY

- Engage in only minimal activity for the remainder of the day after your biopsy. This means you should stand as little as necessary. If possible, you should lie on a couch or in bed for the remainder of the day. It is OK to get up to use the bathroom, etc. Do not engage in any strenuous activity (for example sports). Keeping the knee flexed as much as possible (leg bent) the first 24 hours especially while sleeping, will aid in recovery and reduce stiffness.

ACE WRAP/DRESSING CARE

- Keep the pressure (ace) wrap on for the next 24 hours. (If the wrap is too tight when you go to bed- you can loosen it a little- but do not remove it). During the next 24 hours, use ice packs applied to the biopsy site for 20 minutes every two hours when possible, this will reduce swelling.

- Keep the incision dry and covered with the gauze bandage for at least 48 hours. This means sponge baths (**NO** showers).

- Before you take a shower (after 48 hours), cover the biopsy site with a 2x2 gauze and a waterproof Nexicare (Tegaderm) skin cover to keep the site dry. After the shower remove the wet Tegaderm and replace any wet bandages as necessary.

- Keep the incision site covered for one week while the incision heals. Five to seven days after the biopsy, minor itchiness at the incision site may occur which is normal and a sign of healing. If you have sutures, do not attempt to remove them, Sutures will be removed at your scheduled visit by the Physician one week after the biopsy in the Muscle Biology and Imaging Lab in room 163 Totman. If the physician decides it is necessary to keep the sutures in for a longer period of time, you may be asked to return at a later date to have the sutures removed.

MEDICATIONS

- Take **2** Tylenol (acetaminophen, 1000 mg) immediately after the biopsy and then every 4 to 6 hours if you have pain.

- Do **NOT** take any anti-inflammatory medications (called NSAIDS) like ibuprofen (Advil, Motrin, Nuprin) or aspirin, naproxen (Aleve, Naprosyn) or any aspirin containing drugs such as Alka-Seltzer, Pepto-Bismol, or certain decongestants (such as Dristan) within 4 days of the muscle biopsy. ***All of these medications may increase the risk of bleeding.***

APPENDIX B: Standard Operating Procedures

POST-BIOPSY CONTACTS

- You will come into the lab for several visits post-biopsy. At those visits we will check your incisions to ensure that they are healing as expected.
- On the days that you do not come into the lab, we may call you to check on the progress of your recovery.

WHEN TO CALL WITH CONCERNS

Please call the study coordinator at **ANY** time of day if any of the following occur. (In case of a medical emergency please seek immediate medical assistance.)

- Fever
- Pus or foul smelling drainage
- Severe bleeding
- Severe pain/discomfort
- Stitches, steri-strips or tegaderm come off before 7 days
- Stiffness in the leg
- Swelling/warmth/redness around the incision

IF IN DOUBT PLEASE CALL:

Lab Emergency Phone	**(24hr)**	**(413) 230-9669**
Muscle Biology and Imaging Lab	**Office**	**(413) 545-6072**
Stuart R. Chipkin, M.D.	**Office**	**(413) 545-0089**
Priscilla M. Clarkson, PI	**Office**	**(413) 577-3902**

APPENDIX C: New England American College of Sports Medicine Abstract

Abstract presented at the annual fall meeting of the New England chapter of the American College of Sports Medicine in November, 2010 in Providence, RI

SMOKING AFFECTS MUSCLE RESPONSE TO ECCENTRIC EXERCISE

N Moore; Chipkin, S; Clarkson, PM FACSM
Department of Kinesiology, University of Massachusetts, Amherst, MA

Purpose: Eccentric exercise can result in muscle damage as evidenced by prolonged strength loss, delayed soreness, and inflammation leading to secondary damage. Cigarette smoking is associated with increased systemic inflammation, delayed wound healing, and higher rates of musculoskeletal injury. Performance of eccentric exercise in the presence of existing systemic inflammation may exacerbate exercise-induced muscle damage. Thus, we hypothesized that smokers would have greater strength loss following eccentric exercise. **Methods:** Participants were 19 healthy, sedentary men, 10 smokers (SM) and 9 non-smokers (NS) (23 ±1 years). Subjects performed 100 maximal eccentric contractions with the knee extensors of the non-dominant leg. 48h later, muscle biopsies were taken from the vastus lateralis of both legs (data to be analyzed). Strength of the extensors and flexors was measured pre- and 5 minutes, 1d, 4d, and 9d post-exercise using a Biodex dynamometer. Isometric strength was measured at a knee angle of 70°, and isokinetic strength was measured at angular velocities of 60 and 180°/s. **Results:** During exercise, total work did not differ between NS and SM. *Isometric Extension Strength:* Both NS and SM responded similarly to exercise with significant strength loss ($p<0.05$) that persisted through 4d; at 9d both groups had nearly returned to baseline. *Isometric Flexion Strength:* Because the exercise was performed in the extensors, we expected to find no significant alterations in flexor strength. However, at 4d post-exercise, SM flexion strength decreased to 80% of baseline ($p<0.05$) while NS did not exhibit strength loss. *Isokinetic Strength:* Smokers had lower baseline strength than NS for extension at 180°/s (84.7%, $p<0.05$) and flexion at 60 (72.8%, $p<0.01$) and 180°/s (71.8%, $p<0.01$). Strength changed over time ($p<0.05$) similarly in NS and SM for extension and flexion at both speeds. SM had lower extension strength at all timepoints at 60°/s ($p<0.05$); there were no significant differences between NS and SM for extension at 180°/s or flexion at both speeds. **Conclusion:** Smokers had lower baseline isokinetic strength, and, after eccentric exercise and muscle biopsy, experienced delayed loss of isometric strength in the flexors. These data may suggest an inability to coordinate agonist/antagonist activity in smokers, which may be exacerbated by a heightened inflammatory response.

Supported by a grant from the U.S. Army Medical Research and Materiel Command

APPENDIX D: American College of Sports Medicine Abstract

Abstract submitted for presentation at the national meeting of the American College of Sports Medicine in May, 2011 in Denver, CO

LATE-APPEARING INTRAMUSCULAR HEMATOMA AFTER ECCENTRIC EXERCISE AND MUSCLE BIOPSY: A CASE REPORT

Nina A Moore, Stuart R Chipkin, Priscilla M Clarkson, FACSM
University of Massachusetts, Amherst, MA

Muscle biopsy is commonly used in research. One rare complication is a hematoma that generally develops rapidly within 72h post-biopsy.
PURPOSE: Here we report the unusual case of a late-appearing hematoma following eccentric exercise and muscle biopsy.
METHODS: 10 sedentary, healthy men (22 ± 0.7y) participated in a kinesiology research study. Baseline strength of the knee extensors and flexors (isometric and isokinetic at 60 & 180°/s) was tested followed by an eccentric exercise of the knee extensors of 1 leg—10 sets of 10 maximal effort repetitions at 30°/s with 1' rest between sets. Strength was re-assessed 5', 1, 4, and 9d post-exercise. 2d after exercise, muscle biopsies were taken from the vastus lateralis of both legs. All exercise sessions and biopsies were uneventful. Subjects were directed to limit activity for 1 wk post-biopsy.
RESULTS: The case subject's isometric strength loss at 1d post-exercise (45% extension, 19% flexion) was greater than other subjects (22% and 4%, respectively). At 2d post-biopsy, his isometric strength dropped precipitously (90% extension, 40% flexion), while other subjects regained strength. Similar results were found for isokinetic strength. On 4d post-biopsy the subject spent 5-6h standing for his job. On 5d post-biopsy he experienced transient muscle cramping in the exercised leg. The next evening the subject reported rapidly increasing cramping, pain, and swelling in the exercised leg. Upon admittance to a local hospital, his exercised thigh was swollen by 2.5cm with no indication of infection, fracture, or compartment syndrome. Serum creatine kinase (CK) was elevated at 5,630 U/L (8d post-exercise) with no sign of renal compromise. A diagnosis of intramuscular hematoma was made. After 3d of local care, the subject was released from the hospital. With physical therapy, he returned to normal function within 2 mo.
CONCLUSION: The case subject exhibited profound strength loss and elevated CK suggesting exercise-induced muscle damage. Increased activity on 4d post-biopsy may have further injured damaged tissue, promoting bleeding and hematoma. These data emphasize the importance of limiting activity and maintaining contact with subjects for at least a week after a muscle biopsy.
Supported by a grant from the US Army Medical Research and Material Command

APPENDIX E: Manuscript submitted

Case report of a late-appearing hematoma after eccentric exercise and muscle biopsy

Moore, Nina A.; Chipkin, Stuart R.; Clarkson, Priscilla M.

Department of Kinesiology, Totman Building, University of Massachusetts, Amherst, MA

Address for correspondence: Nina A. Moore, 110 Totman Building, University of Massachusetts, Amherst, MA 01003; camoore@kin.umass.edu; telephone: 413-545-6072, fax: 413-545-2906.

RUNNING TITLE: Late hematoma after exercise and biopsy

DISCLOSURE OF FUNDING: U.S. Army Medical Research and Material Command

APPENDIX E: Manuscript submitted

Abstract:

PURPOSE: A rare complication of a muscle biopsy is a hematoma that generally develops within 72 h. Here we report an unusual case of a late-appearing hematoma following eccentric exercise (ECC) and muscle biopsy.

CASE SUMMARY: During a study, 10 sedentary, healthy men (22 ± 0.7 y) performed a unilateral knee extensor ECC. 2 d later, muscle biopsies were taken from both legs (vastus lateralis). Knee flexion and extension strength was assessed pre- and 5 min, 1, 4, and 9 d post-exercise. 1 d post-exercise the case subject's isometric strength loss was greater (45% extension, 19% flexion) than other subjects (mean±SEM= 22±6% and 4±5%, respectively). 2 d post-biopsy, his strength dropped precipitously (90% extension, 40% flexion), while other subjects regained strength. 4 d post-biopsy the subject spent 5-6 h standing for his job. At 5 d post-biopsy he experienced transient muscle cramping in the exercised leg. The next evening he reported rapidly increasing cramping, pain, and swelling. Upon admittance to a local hospital (6 d post-biopsy), his exercised thigh was swollen by 2.5 cm with no indication of infection, fracture, or compartment syndrome. Serum creatine kinase (CK) was elevated (5,630 U/L) without renal compromise. A diagnosis of intramuscular hematoma was made. After 3 d of local care, the subject was discharged. With physical therapy, he reached normal function within 2 mo.

CONCLUSION: The case subject's strength loss and elevated CK suggest exercise-induced muscle damage. Increased activity post-biopsy may have further injured damaged tissue, promoting a hematoma. These data emphasize the importance of limiting activity for at least a week after a muscle biopsy.

Key words: rhabdomyolysis, muscle damage, creatine kinase, strength loss

APPENDIX E: Manuscript submitted

Paragraph 1 **Introduction:**

Duchenne first developed the needle muscle biopsy technique in the 1860s as a means to characterize muscular dystrophy (2). It was not until 1962 that Bergström modified this technique and created the Bergström biopsy needle (1) that is still commonly used to take muscle samples today (3, 7, 9, 13-17). In the 1970s, the muscle biopsy technique became a popular tool in exercise science research to study the effects of exercise and disuse at the tissue level. Although considered safe, there are complications associated with muscle biopsies that range from mild to serious and include prolonged pain, infection, numbness, and hematoma (4, 8, 12).

Paragraph 2 Highstead et al. (8) evaluated the incidence of hematoma, bleeding, and infection after a muscle biopsy in 362 research studies spanning nine years. Of the 1,301 muscle biopsies performed, 1,288 were taken from the vastus lateralis; all were taken using a 5 mm Bergström needle. Only 18 incidents of hematoma were reported, which was 1.4% of all biopsies. At the University College Hospital in London, Ontario, a similar biopsy technique used over 10 years resulted in 3 hematomas in 800 muscle biopsies—this represents less than 0.5% of the total biopsies collected during muscle disorder diagnostic procedures (5).

Paragraph 3 Without treatment, a hematoma is typically reabsorbed over time (6). However, pressure caused by increased swelling from intramuscular hematoma can be quite painful. In rare cases, surgery is needed to remove the hematoma (10). Following contusion injury, a hematoma develops rapidly—usually within the first day of injury (10, 11)—and can take weeks to months for the patient to return to normal function. Although the time course of hematoma development

following a muscle biopsy has not been published, it is generally accepted that, like following contusion injury, hematoma develops rapidly—within the first 72 hours—after a muscle biopsy.

Paragraph 4 Here we present an atypical case of a late-appearing hematoma following eccentric exercise and a muscle biopsy. The unusual time course of this case is important because it occurred when post-biopsy pain has abated; at this point vigilance regarding the biopsy site is often reduced, and subjects tend to return towards normal activity levels. The subject has provided his consent for the publication of his case.

Paragraph 5 **Case Report:**

A 20-year-old male subject was participating in a research study involving knee extension eccentric exercise and a muscle biopsy of the vastus lateralis. The subject completed a medical history questionnaire and gave written informed consent as approved by the University of Massachusetts Amherst Institutional Review Board prior to participation in the study (University of Mass Protocol #108-1099R). He was sedentary but generally healthy, with no history of resistance training or lifting/lowering of heavy objects within the past six months. There was no history of musculoskeletal, metabolic, cardiovascular, hematological, or other chronic diseases; the subject had never smoked cigarettes. The subject was not taking any dietary supplements to enhance muscle size or alter body weight and did not take any medications. The subject consumed approximately 3 alcoholic drinks per week. According to the study protocol, the subject refrained from consuming alcohol- and caffeine-containing products and anti-inflammatory medications during the course of the study.

Paragraph 6 Muscle strength of the knee extensors and flexors was measured, and a bout of strenuous knee extensor eccentric exercise was performed with the non-dominant leg using a Biodex System 4 dynamometer (Biodex Medical Systems, Shirley, NY). Maximal isometric strength was measured at 70 degrees of flexion for both extension and flexion. Three repetitions were performed, and each was separated by one minute of rest.

Paragraph 7 The exercise consisted of 10 sets of 10 maximal eccentric contractions of the knee extensors at 30 degrees per second. Each repetition was separated by 10 seconds of rest, and there was one minute of rest between each set. The subject was permitted to drink water *ad libitum*. After five minutes of rest, the subject repeated the strength measures. Figure 1 presents the strength loss for the case subject and the other subjects in the same study group assignment. For all subjects, there was an immediate loss of strength within expected ranges. At the time of exercise, the case subject did not complain of undue fatigue or pain. All subjects were instructed to maintain normal hydration in the days following exercise. The next morning (1 d post-exercise) strength was again measured; for the case subject, strength had decreased compared to the post-exercise measure while strength increased for the other subjects (Figure 1).

Paragraph 8 Muscle samples were taken from the vastus lateralis of both legs using a Bergström needle biopsy two days after the exercise session. The procedure was uneventful. The skin was cleaned with betadine and infused with 2% lidocaine (McKesson, San Francisco, CA). A 1.5 cm incision was made and additional lidocaine was administered. Throughout the procedure pressure was placed to control superficial bleeding. Following a nick in the muscle fascia, a Bergstrom needle was used with suction to obtain muscle samples from the vastus

lateralis. 4-0 prolene was used to place 3 sutures. The procedure was repeated on the opposite leg. No excess bleeding or pain was noted during or immediately after the procedure.

Paragraph 9 Two days after the muscle biopsy procedure, strength had decreased profoundly in the case subject (Figure 1) as compared with the other subjects. When questioned by staff, he did report feeling more weak than usual, although his movement and regular activity were not impaired, and he was not experiencing pronounced soreness. Visual inspection by the study investigator revealed no swelling or bruising present in either leg, and there were no signs of infection at the biopsy incisions. The investigator contacted the study subjects every evening by telephone to monitor their recovery; during these calls the case subject reported continued improvement of range of motion and decreased soreness with each successive day. A timeline of events is presented in Table 1.

Paragraph 10 On 4 d post-biopsy (6 d post-exercise), in the course of his work, the subject spent approximately 5-6 h standing, with no ill effects at the time. However, the next evening (5 d post-biopsy, 7 d post-exercise) he experienced transient muscle cramping in the exercised leg. These experiences were reported to the study investigator on the evening of the sixth day post-biopsy (8 d post-exercise) during the regular follow-up telephone call. The subject was not experiencing any cramping at the time of this telephone conversation. Thirty minutes after the initial conversation, the subject contacted the study investigator by telephone and reported rapidly increasing muscle pain, cramping, and swelling of the leg. The subject reported no change in urine color. The subject was initially advised to attempt symptom alleviation with acetaminophen, compression, elevation, and warm compresses. When the symptoms did not

abate, the subject phoned the study investigator who, along with the study physician, recommended that he go to the closest emergency department to seek treatment.

Paragraph 11 When he arrived at the emergency department, the case subject's blood pressure and pulse were elevated at 148/98 mm Hg and 120 beats per minute, respectively; temperature, O_2 saturation, and respiratory rate were normal. Examination by the admitting physician revealed swelling of the leg with no sign of infection or other notable health concerns. Initial laboratory results (Table 2) showed a slightly elevated white blood cell count, elevated neutrophils, and a slight elevation of C-reactive protein (0.8 mg/dL); low red blood cell count, hemoglobin, and hematocrit; and normal blood clotting parameters (prothrombin time, INR, and activated partial thromboplastic time). Blood creatine kinase (CK) activity upon admittance was elevated at 5,630 U/L. CK MB levels were within normal ranges. All measures for urinalysis were normal including lack of hematuria.

Paragraph 12 The subject was admitted to the hospital for observation, and intravenous fluids and pain medication were given. An orthopedic surgeon examined the subject and differential diagnosis focused on compartment syndrome, rhabdomyolysis, infection, and hematoma. Compartment syndrome was eliminated due to the lack of excruciating pain, absence of severe tension, and the subject's leg could be flexed passively without discomfort to 30-45 degrees of flexion. Lack of hematuria coupled with relatively modest CK elevation excluded diagnosis of clinically relevant rhabdomyolysis (i.e. danger of renal failure). X-ray of the thigh showed soft tissue swelling but normal bone mineralization and shape; no air or gas was present in the soft tissue. These findings excluded diagnoses of fracture or anaerobic infection. To assess the soft

tissue swelling, the orthopedic surgeon measured the subject's leg circumference with a tape measure. The non-dominant leg measured 44.45 cm in circumference at 15.24 cm above the superior pole of the patella as compared to 41.91 cm in the dominant, non-exercised leg. The final diagnosis was presumed to be intramuscular hematoma. The subject was also found to be anemic, which may have been pre-existing or secondary to the hematoma, intravenous fluids, or the combination.

Paragraph 13 While in the hospital, the subject received local care (rest, ice, and continued observation), acetaminophen with oxycodone (325 mg – 5 mg PO q 4 hours PRN), and morphine (2 mg IV q 2 hours PRN) for pain. In addition, the subject received intravenous fluids including potassium and multi-vitamin supplements. During hospitalization, hematocrit and hemoglobin levels did not decrease further, swelling did not worsen, and pain decreased. The subject was discharged after 3 days and instructed to use crutches, rest, and continue icing. With physical therapy, the subject regained full range of motion and normal mobility within the following month. The subject then returned to normal activity, including some recreational jogging, with no additional adverse effects.

Paragraph 14 **Discussion:**

This case is of interest due to the late-appearing nature of the hematoma and marked decrease in strength following eccentric exercise. Bleeding and subsequent hematoma most commonly occur in the days immediately following injury (10, 11). In this case study, at two days post-biopsy the subject showed no sign of swelling, bruising, or unusually high soreness. However, he had a precipitous drop in strength (Figure 1). Over the next three days he reported that his soreness was

decreasing and he was returning to his pre-biopsy level of function. On the fourth day post-biopsy the subject spent the majority of his day standing, while in previous days he had been mostly sedentary. It was not until after this day that the subject developed symptoms leading to hospitalization.

Paragraph 15 Strength loss is considered a good indicator of muscle damage in response to eccentric exercise (18). In the case reported here, the reduction in strength post-exercise prior to the development of the hematoma raises the possibility of significant damage to muscle fibers that resulted in intramuscular bleeding at the biopsy site. In a recent study in our laboratory using the same exercise with 35 subjects, 33 had a peak CK below 5,000 U/L and two had a peak above 5,000 U/L. For the case subject, the unusual strength loss in combination with a CK activity of 5,630 U/L may indicate a greater amount of exertional muscle damage compared with the other subjects; this muscle damage could have pre-disposed him to further bleeding in response to prolonged standing (4 d post-biopsy) generating a painful, debilitating hematoma that required hospitalization.

Paragraph 16 While rare, there is a risk of delayed hematoma following a muscle biopsy, and this risk may be exacerbated by damage from performance of strenuous exercise in the days prior to the biopsy. It is important to maintain contact with subjects until at least one week post-biopsy and stress that subjects be cautious in performing unaccustomed exercise during this time.

APPENDIX E: Manuscript submitted

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FIGURE CAPTIONS:

Table 2 footnote: Hospital reference values are provided in parentheses for each measure. An asterisk (*) indicates an abnormal measurement. MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = red blood cell distribution width; MPV = mean platelet volume

Figure 1: Peak isometric strength for extension (a) and flexion (b), presented as a percentage of baseline, following eccentric exercise of the leg (pre-exercise, immediately post-exercise, 1, and 4 days post-exercise). Closed bars indicate the case report subject. The open bars indicate the average of all other subjects in the study (9 subjects).

ACKNOWLEDGMENTS: Parent study funded by U.S. Army Medical Research and Material Command

CONFLICT OF INTEREST: none

APPENDIX E: Manuscript submitted

FIGURES:

Table 1: Schedule of Events

Days post-exercise	Days post-biopsy	Comments	Contact
-	-	Exercise procedure normal	Lab Visit
2	-	Biopsy procedure normal	Lab Visit
3	1	Reported no undue pain, no report of unusual weakness	Telephone Call
4	2	Significantly lower strength than 1 d post-exercise and compared with other subjects; reported no undue pain	Lab Visit
5	3	Symptoms abating	Telephone Call
6	4	Subject spent 5-6 h standing; symptoms abating	Telephone Call
7	5	Evening: transient cramping in exercise leg, resolved when lying down	Telephone Call
8	6	Evening: increasingly intense cramping and pain in exercised leg; went to emergency room at hospital and admitted	Telephone Call

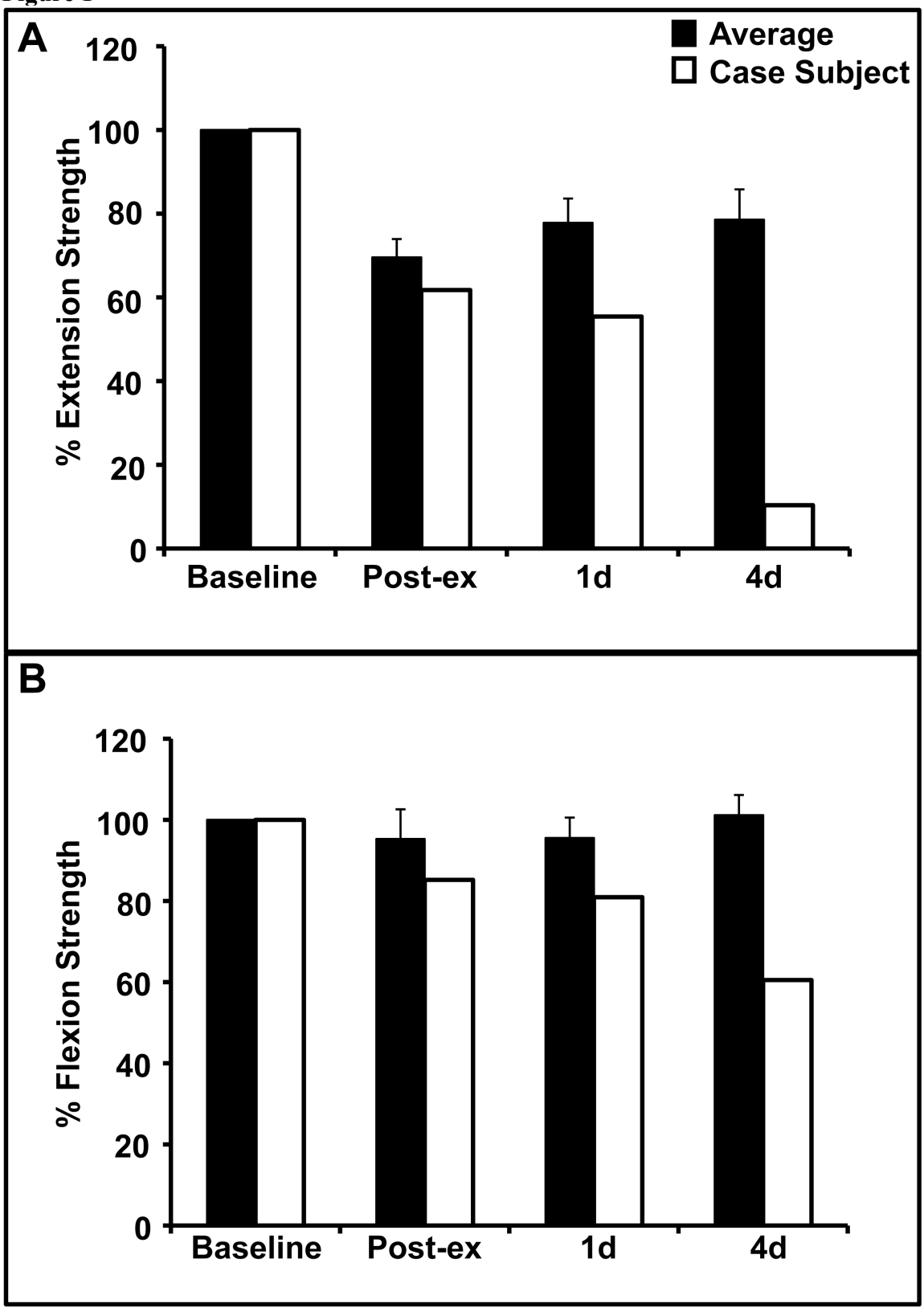
APPENDIX E: Manuscript submitted

Table 2: Laboratory Results

	Day					
	1				2	3
	1:00 AM	9:00 AM	3:00 PM	10:00 PM	6:30 AM	6:30 AM
Complete Blood Count with Automated Diff						
White blood cell count (3.4-11.2 K/uL)	12.7*	8.8			7.4	
Red blood cells (4.5-5.5 M/uL)	3.73*	3.52*			3.40*	
Hemoglobin, Whole Blood (13.0-17.0 g/dL)	10.5*	9.9*		9.6*	9.6*	9.9*
Hemoglobin A1C, Whole Blood (4.3-5.8%)			5.7			
Hematocrit, Whole Blood (40.0-51.0%)	30.2*	28.4*	27.9*	27.6*	28.0*	28.8*
MCV (79-98 fL)	81.0	80.7			82.4	
MCH (27.0-34.8 pg)	28.2	28.1			28.2	
MCHC (31.5-36.0 g/dL)	34.8	34.9			34.3	
RDW (10.8-14.6%)	12.1	12.2			12.6	
Platelet count (130-400 K/uL)	204	227			242	
MPV (7.2-10.5 fL)	9.5	9.5			9.9	
Neutrophils, segmented (45.3-77.7%)	79.0*					
Absolute neutrophil count (1.4-7.7 K/uL)	11.4*					
Lymphocytes (12.3-39.7%)	6.0*					
Lymphocyte absolute (0.6-3.2 K/uL)	0.8					
Monocytes (4.1-12.8%)	4.0*					
Absolute monocyte (0.1-0.6 K/uL)	0.5					
Eosinophils (0.0-7.2%)	0					
Eosinophil absolute 0.01-0.5 K/uL)	0.0*					
Basophils (0.0-2.8%)	0					
Absolute basophils (K/uL)	0.01					
Immature granulocytes (0.0-0.5%)	0.2					
Absolute immature granulocytes (0.00-0.03 K/uL)	0.03					
Basic Metabolic Panel						
Glucose, serum (77-99 mg/dL)	165*					
Blood urea nitrogen, serum (6-19 mg/dL)	17					
Creatinine, serum (0.5-1.5 mg/dL)	0.6					
Sodium level (133-145 mEq/L)	127*	138			134	
Potassium level (3.3-5.1 mEq/L)	3.2*	3.7			4.1	
Chloride level serum (96-108 mEq/L)	93*	102			99	
Carbon dioxide level (21-35 mEq/L)	25	25			30	
Anion gap (10-20 mEq/L)	12	15			9*	
Calcium, serum (8.4-10.3 mg/dL)	8.6					
Glomerular filtration rate	>60					

APPENDIX E: Manuscript submitted

Figure 1



APPENDIX F: New England American College of Sports Medicine Abstract

Abstract accepted for presentation at the annual fall meeting of the New England chapter of the American College of Sports Medicine in November, 2011 in Providence, RI

Smoking and Muscle Damage: Blunted Changes in Muscle NFkB Activity after Eccentric Exercise

N Moore; Chipkin, S; Clarkson, PM FACSM
Department of Kinesiology, University of Massachusetts, Amherst, MA

Purpose: For unknown reasons, smoking increases the risk for musculoskeletal injury and prolonged healing. We used an eccentric exercise (muscle damaging) model to determine potential mechanisms to explain this risk. Muscle regeneration after eccentric exercise is characterized by alterations in gene expression leading to proliferation and differentiation of muscle progenitor cells (MPCs). NFkB is a transcription factor that stimulates MPC proliferation and differentiation via canonical and non-canonical pathways, respectively. We hypothesized that smokers (SM) would exhibit altered NFkB signaling after eccentric exercise, potentially explaining impaired muscle regeneration and increased risk for injury. **Methods:** Healthy, sedentary men, 10 SM and 9 non-smokers (NS) (23±1y), performed 100 maximal eccentric contractions with the knee extensors of the non-dominant leg (EX). 48h later, muscle biopsies were taken from both legs (vastus lateralis; non-exercised leg served as the control (CON)). A custom-designed PCR array (SABiosciences, Frederick MD) was used to compare mRNA expression in the EX vs CON leg. Muscle NFkB activity was quantified with antibodies against p65 (canonical) and Rel-B (non-canonical) (Active Motif, Carlsbad, CA). Data are mean±SEM. **Results:** Gene expression change with EX was significantly different between NS and SM in 7 of 44 genes examined. The largest difference between NS and SM was IKKa expression with 2.3-fold upregulation vs -2-fold downregulation, respectively ($p=0.026$). Since IKKa activity can activate NFkB signaling, we quantified NFkB activity. In the CON leg, SM activity was lower than NS for p65 (0.023±0.01 vs 0.06±0.008 OD) but not Rel-B. In response to EX, NS had reduced p65 signaling (0.06±0.008 CON vs 0.01±0.005 EX) while SM had no alteration (0.023±0.01 vs 0.022±0.01). Rel-B signaling was increased in NS with exercise (0.041±0.02 CON vs 0.095±0.04 EX) but not in SM (0.03±0.01 CON vs 0.045±0.01 EX). **Conclusion:** At 48h post-exercise, there was an increase in IKKa mRNA for NS but a decrease for SM. Rel-B activity was increased in NS while p65 activity was decreased, suggesting an impairment in IKKa activation of these NFkB pathways. Decreased IKKa mRNA and overall suppression of NFkB activity suggests blunted myogenic signaling in SM. Together, these data indicate a mechanism through which smoking may impair muscle regeneration. Supported by a grant from the U.S. Army Medical Research and Materiel Command

APPENDIX G: American College of Sports Medicine Abstract

Draft of abstract to be submitted for presentation at the national meeting of the American College of Sports Medicine in May, 2012 in San Francisco, CA

Smokers Exhibit Blunted Changes in Muscle NFkB Activity after Eccentric Exercise

Nina A. Moore, Stuart R. Chipkin, Priscilla M. Clarkson, FACSM.
Department of Kinesiology, University of Massachusetts, Amherst, MA

Purpose: For unknown reasons, smoking increases the risk for musculoskeletal injury and prolonged healing. We used an eccentric exercise (muscle damaging) model to determine potential mechanisms to explain this risk. Eccentric contractions (ECC) result in alterations to gene expression leading to proliferation and differentiation of muscle progenitor cells (MPCs) and subsequent muscle regeneration. The transcription factor NFkB can stimulate MPC proliferation and differentiation via canonical and non-canonical pathways, respectively. We hypothesized that smokers (SM) would have altered NFkB signaling after ECC, potentially explaining impaired muscle regeneration and increased risk for injury. **Methods:** Healthy, sedentary men, 10 SM and 9 non-smokers (NS) (23±1y), performed 100 maximal ECC with the non-dominant knee extensors (EX). 48h later, muscle biopsies were taken from both legs (vastus lateralis; non-exercised leg served as the control (CON)). A custom-designed PCR array was used to compare mRNA expression between legs. NFkB activity was quantified with antibodies against p65 (canonical) and Rel-B (non-canonical). Data are mean±SEM. **Results:** Gene expression change with EX was significantly different between NS and SM in 7 of 44 genes examined. The largest difference between NS and SM was IKKa expression with 2.3-fold upregulation vs -2.0-fold downregulation, respectively ($p=0.026$). Since IKKa activity can activate NFkB signaling, we quantified NFkB activity. In the CON leg, SM activity was lower than NS for p65 (0.023±0.01 vs 0.06±0.008 OD) but not Rel-B. In response to EX, NS had reduced p65 signaling (0.06±0.008 CON vs 0.01±0.005 EX) while SM had no alteration (0.023±0.01 vs 0.022±0.01). Rel-B signaling was increased in NS with exercise (0.041±0.02 CON vs 0.095±0.04 EX) but not in SM (0.03±0.01 CON vs 0.045±0.01 EX). **Conclusion:** In response to ECC: 1) IKKa mRNA was increased in NS but decreased in SM; 2) Rel-B activity was increased in NS but not in SM; and 3) p65 activity decreased in NS while there was no change in SM. These data suggest impaired IKKa activation of these NFkB pathways and blunted myogenic signaling in SM. Together, these data indicate a mechanism through which smoking may impair muscle regeneration. Supported by a grant from the US Army Medical Research and Materiel Command

Appendix H: Western blotting

Blot layouts were designed as in Appendix H figure 1. Six non-smokers (at left) and 6 smokers (right) were tested. One non-smoker was removed from analyses due to problems with sample integrity. Analyses were performed by normalizing densities to the density of the corresponding loading control (GAPDH).

Figure 1: Blot layout
L = ladder, N = non-smoker, S = smoker, C = control leg, X = exercise leg.

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Smoke Status	L	N	N	N	N	N	N	N	N	N	N	N	N	L	S	S	S	S	S	S	S	S	S	S	S	S
Subject		1	1	2	2	3	3	4	4	5	5	6	6		1	1	2	2	3	3	4	4	5	5	6	6
Leg		C	X	C	X	C	X	C	X	C	X	C	X		C	X	C	X	C	X	C	X	C	X	C	X

Figure 2: α-actin

Figure 3: phosphorylated ERK1/2

Figure 4: total ERK1/2

Figure 5: Caspase-3

Figure 6: MMP9

Figure 7: FGF

Figure 8: GAPDH as loading control for Actin, MMP9, FGF, and Caspase-3

Figure 8: GAPDH as loading control for pERK1/2 and total ERK1/2